JOURNAL OF THE NATIONAL CANCER INSTITUTE

February 1958
Volume 20 • Number 2

A Publication of the
U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

JOURNAL OF THE NATIONAL CANCER INSTITUTE

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NATIONAL INSTITUTES OF HEALTH • Public Health Service

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A speculative article by Dr. George Crile, Jr., is presented in the following pages. The Board of Editors of the Journal of the National Cancer Institute would like to take this opportunity to repeat the invitation for thoughtful speculative papers on cancer—its nature, origin, and development. This invitation is prompted by a desire to stimulate informed theorizing by those involved in cancer research or those who have considered the field from some other vantage point of science, since it is our conviction that speculative summaries have heuristic as well as historical and philosophical value.

Discussions of published hypotheses are also invited; they should be prepared according to the "Suggestions to Authors" on the inside back cover and submitted along with pertinent bibliography.

The chief mission of the Journal remains as before: the publication of original contributions to cancer research through laboratory and clinical investigations and statistical studies. The publication of any paper by the Journal does not imply its endorsement by the National Cancer Institute.

A Speculative Review of the Role of Endocrine Imbalances in the Genesis of Certain Cancers and Degenerative Diseases [1]

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This paper is a review of experimental and clinical evidence that prolonged stimulation of target cells by their trophic hormones is one of the causes of cancer. In this thesis the concept of trophic hormones and target cells is extended beyond the conventional limits of the endocrine system.

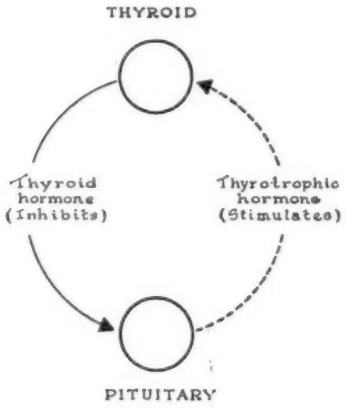
I. Proved Role of Endocrine Imbalances in Production of Cancer in Endocrine Glands and Their Target Organs

The administration or withdrawal of hormones can stimulate or control the growth of certain cancers of the human breast, prostate, and thyroid. In animals, too, it has been shown (*1–5*) that the growth of cancers can be stimulated by disturbing endocrine balances and that often these cancers can be controlled by restoring normal endocrine balance. Prolonged and excessive stimulation of a target organ by a trophic hormone first causes hyperplasia of the animal's target organ, then formation of benign nodules, and then cancers. At first these cancers may be dependent for their continued growth on the persistence of the endocrine imbalance that produced them but, finally, if the imbalance persists, autonomous cancers develop and can grow in animals in which there is no longer any endocrine imbalance; for example, the production of cancer of the thyroid by feeding thiouracil (*5*).

When thiouracil is fed to rats and the output of thyroid hormone is suppressed, the pituitary's output of thyrotrophic hormone is increased (text-fig. 1). Stimulated by the specific growth hormone thyrotrophin, the thyroid goes through progressive changes of hyperplasia, formation of benign nodules, and formation of metastasizing cancers that at first are dependent for their growth on the presence of an excess of thyrotrophic hormone. Finally, if the dependent cancers are continuously stimulated by thyrotrophic hormone, they become autonomous and can grow in animals receiving no thiouracil. Most of these changes can be induced by any agent that reduces the output of thyroid hormone, including antithyroid drugs, radioactive iodine, dietary deficiency of iodine, or

[1] Received for publication August 5, 1957.

subtotal thyroidectomy combined with administration of carcinogens. But administration of thyroid hormone suppresses the output of thyrotrophic hormone and protects the thyroid against the changes induced by all of these agents (*3*). It is therefore not the thiouracil or the I^{131} that is the carcinogen but the endogenous growth-stimulating thyrotrophic hormone. In rats or mice, similar stimulation of endocrine or target organs by endogenous trophic hormones may give rise to cancers in the ovaries, testes, pituitaries, breasts, seminal vesicles, kidneys, or livers (*4*).



TEXT-FIGURE 1.—*The Thyroid-Pituitary Axis.* Thyrotrophic hormone from the pituitary stimulates the thyroid, and thyroid hormone inhibits the production of thyrotrophic hormone.

II. Possible Role of Endocrine or Chemical Imbalances in Production of Cancer in "Nonendocrine" Organs

Although most of the cancers induced in experimental animals have been produced by disturbing the function of endocrine organs, Rous and Kidd (*6*) and others have produced cancers by applying chemical carcinogens to the skin. Bielschowsky (*3*), in discussing these cancers, observed that there appear to be no fundamental differences between the tumors induced by application of exogenous carcinogens and those produced by prolonged stimulation of endocrine glands. In each case there is ". . . at first diffuse hyperplasia, followed by nodular hyperplasia, and finally by frankly neoplastic growth." These observations raise the question of whether the chemical carcinogens act directly on the cells of the skin or whether they act indirectly, as thiouracil does in cancer of the thyroid, by increasing the output of an endogenous growth-stimulating chemical.

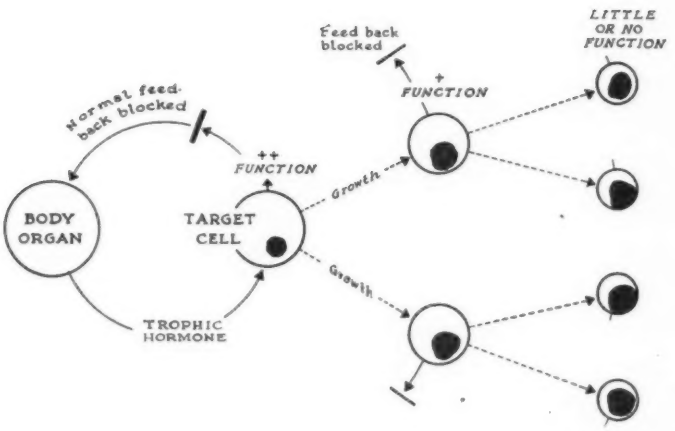
According to Furth (*1*), it has long been postulated that every cell in the body gives rise to substances that affect adjacent cells, but how the optimum number of various types of cells is maintained is a problem common to physiology and cancer research. As early as 1895, Ribbert (*7*) suggested that a malignant change can originate in a loss of the normal

restraints that are exerted on the growth of cells by the cells adjacent to them. Later Weiss (*8*) proposed that synthesis of the protoplasm of an organ results in the formation of diffusible compounds capable of interfering with the further synthesis of that type of protoplasm. As these diffusible substances accumulate in the hormonal pool and reach critical concentrations, growth ceases. Partial removal of an organ reduces the amount of the diffusible compound that the organ makes, and compensatory growth of the rest of the organ ensues. In support of this hypothesis, Weiss showed that the addition of an extract of an organ to an embryo in culture medium reduces the capacity for differentiation in the homologous organ.

Weiss and Ribbert's thesis that cells or products of cells have a restraining effect on one another's growth is like Osgood's (*9*), which assumes that the basic cause of each cell-type of cancer is a deficiency of a specific substance that controls the growth of normal cells of that type. In Osgood's concept, the failure of the cell to make the necessary restraining substance is dependent upon a genetic change in which the cell loses its ability to differentiate and live. The resulting deficiency of mature cells results in insufficient production of the inhibitors of cell division, growth is stimulated, and cancer ensues. This basic concept is in harmony with the observations of those who have produced cancers in animals by inducing endocrine imbalances and subjecting target organs to excessive stimulation by trophic hormones. Osgood's hypothesis, that the failure of cells to mature results in their failure to secrete a growth-restraining chemical, should be studied to determine whether the initial change in the cells is truly genetic or whether it is the result of chemical stimulations by trophic hormones. Perhaps, under the influence of excessive stimulation, the cells of a target organ divide so often that they no longer mature enough to develop function. If an entire tissue were to expend most of its energy on growth instead of on function, it is possible that the feed-back mechanism that controls growth might be paralyzed and a vicious circle of abnormal growth might be established. Ultimately following prolonged exposure to a growth-promoting substance, genetic changes in the cells might ensue (text-fig. 2).

III. Hyposecretion of "Signal Substances" and Compensatory Hypersecretion of Trophic Hormones as a Possible Cause of Certain Cancers

As knowledge of physiology and biochemistry increases, it is found that many organs besides those of the conventional endocrine system have endocrine functions. It is beginning to appear that the endocrine system may be synonymous with the living parts of the entire body and that each type of cell makes a specific chemical substance that controls the output of a trophic hormone. Just as the output of thyroid hormone controls the production of thyrotrophic hormone, a specific chemical made by a skin cell may control the secretion of a specific trophic or "skin-growth" hormone made by another type of cell.



TEXT-FIGURE 2.—*Diagrammatic Representation of the Manner in Which Continuous Stimulation by a Trophic Hormone Could Result in Progressive Loss of Function of the Target Organ and in the Establishment of a Vicious Circle of Growth.* When a trophic hormone stimulates a target cell, the cell is stimulated not only to increase its function but also to grow and divide. As the trophic hormone continues to stimulate the descendants of the initial target cell, the growth and division of these cells is further stimulated to the point that they divide at a younger age, perhaps before they have differentiated and gained full function. Finally, if the feed-back mechanism is blocked, the target cells under excessive stimulation by trophic hormones may divide so early that they never attain function. If this should take place, the output of the trophic hormone could not be controlled by removal of the agent that originally blocked the function of the target cell. Thus an excessive stimulus to growth could abolish the function of the target cell, paralyze the feed-back control mechanism, and establish a vicious circle of growth.

In regard to the thyroid the specific chemical made by the thyroid cell has a proved physiologic function, and the effects of its hormone are easily recognized by their gross effects on the body. But the breast, in contrast to the thyroid, is a gland of external secretion whose primary function is to make milk. If, in addition to this external secretory function, the breast has an internally secreted hormone, this hormone may have no physiologic function except to control the output of a trophic substance that stimulates the growth of the breast. Perhaps the skin, whose main function is to protect the underlying tissues, also secretes a hormone that indicates to other cells whether the skin is intact or injured and in need of repair. Such "self-identifying" hormones (*10*) could be called "signal substances." The sole function of the breast's "signal substance" or the skin's "signal substance" may be to indicate to other cells whether or not there is enough breast tissue or enough skin tissue. The "signal substance" of the breast might help control the output of the mammotropic hormone of the pituitary, which, in the presence of estrogens, is known to regulate the growth of the breast.

According to this chemical concept of the control of cellular growth, not only each endocrine gland but also each gland of external secretion,

and each type of cell in the body, may make a hitherto unrecognized "signal substance" whose sole function is to control the output of a trophic hormone made by some other type of cell. Thus, the number of trophic hormones, instead of being limited to the few that we recognize in the pituitary, may equal the number of types of cells in the body.

There is reason to believe that "signal substances" do exist, for how else can we explain the growth and then the cessation of growth of an organ like the breast. If there were no "signal substance" in the breast, how could the pituitary "know" whether the bulk of breast tissue in a maturing girl were adequate and how could it control the secretion of the mammotropic hormone which stimulates the growth of the breast? Even though the breast and prostate are thought of as organs of external secretion, their growth is known to be under the control of the endocrine system. Perhaps in these organs, just as in the recognized endocrine organs, there is a feed-back system that controls their growth. Skin, gastric and intestinal mucosa, blood cells, and, in fact, each type of cell may make a specific "signal substance" which indicates the presence or absence of a sufficient number of cells of that type.

Since one of the characteristics of cancer is excessive growth, it is possible that overstimulation of cells by specific growth hormones is one of the basic causes of cancer. Experiments on the known endocrine axes show that excesses of trophic hormones result in the development of cancers in target organs. The sequence of events is 1) failure of an organ to secrete enough of its hormone or "signal substance," 2) a compensatory increase in output of a trophic, growth-stimulating chemical, and 3) the development of malignant changes in the overstimulated target cells. In this concept, disturbances in the function of hitherto unrecognized endocrine axes may be responsible for the growth of many cancers.

IV. Possibility That Certain Degenerative Diseases Are the Result of Hypersecretion of "Signal Substances" and Consequent Hyposecretion of Trophic Hormones

All known endocrine glands are subject not only to states of hypofunction but also to the development of hyperfunctional states that produce specific diseases like hyperthyroidism. If we assume that each type of cell in the body has an endocrine function and makes a "signal substance" that controls its growth, what would be the result of hypersecretion of such a substance?

In most endocrine glands, small autonomously functioning adenomas cause recognizable disturbances in the hormonal balance of the body. In addition, the hormones made by such tumors suppress the output of trophic hormones and cause atrophy and hypofunction of the rest of the gland.

Occasionally in nonendocrine tissues, just as in the recognized endocrine glands, localized groups of hyperfunctioning cells may exist. If they do, hypersecretion of a "signal substance" might inhibit the output of a specific trophic hormone and the cells in question might atrophy or at

least be so weakened that they would be susceptible to secondary infection and to involutional changes resulting from the withdrawal of their trophic hormone. Perhaps hypersecretion of a "signal substance" by some of the cells of the skin is the cause of certain dermatitides, pemphigus, and other diseases of the skin. Perhaps ulcerative colitis, regional enteritis, arthritis, cirrhosis of the liver, some types of nephritis, pancreatitis, gastritis, and even gastric ulcer are caused by oversecretion of specific "signal substances" and the resultant suppression of trophic hormones. There is a large category of diseases of unknown etiology unrelated to infection or neoplasia. These diseases have one characteristic in common, the cells of the organ affected seem poorly sustained, and often secondary infections or chemical disturbances complicate the picture. These diseases may be the result of hypersecretion of the same chemicals that, when deficient, stimulate the production of an excess of trophic hormones and result in the growth of cancers.

V. Clinical and Laboratory Evidence in Support of the "Signal Substance" Theory

A. Clinical Evidence

There is clinical evidence to suggest that, in man, disturbances in endocrine axes affect the growth of certain cancers and in some cases may be the actual cause of malignant change.

1) *Cancer of the thyroid.*—Most benign goiters, except those that occur in subacute thyroiditis and Graves' disease, are thought to be the result of thyroid failure and the compensatory overproduction of thyrotrophic hormone (*11*). Most of them can be prevented by feeding thyroid hormone.

It is more difficult to prove that overproduction of thyrotrophic hormone is the cause of cancer of the thyroid, but the following clinical observations can be explained by assuming that an excessive production of thyrotrophic hormone, if not the cause of certain cancers of the thyroid, is at least a factor that stimulates their growth:

a) The relatively high incidence of clinical cancer in nontoxic nodular goiters. (The thyroids of patients with such goiters tend to be hypofunctional and thus may stimulate the production of thyrotrophic hormone.)

b) The low incidence of clinical cancer in toxic nodular goiters. (In these glands autonomous secretion of thyroid hormone by adenomas may suppress the production of thyrotrophic hormone and inhibit the growth of thyrotrophin-dependent cancers.)

c) The spontaneous remissions observed in the growth of certain follicle-forming cancers when they enlarge and make enough thyroid hormone to inhibit the formation of thyrotrophic hormone.

d) The rapid spread of certain cancers when hypothyroidism is induced by thyroidectomy, treatment with I^{131}, or feeding thiouracil (*12*).

e) The frequent control of the growth of differentiated thyroid cancers by feeding thyroid, and the occasional disappearance of such tumors when thyroid is fed (*12*).

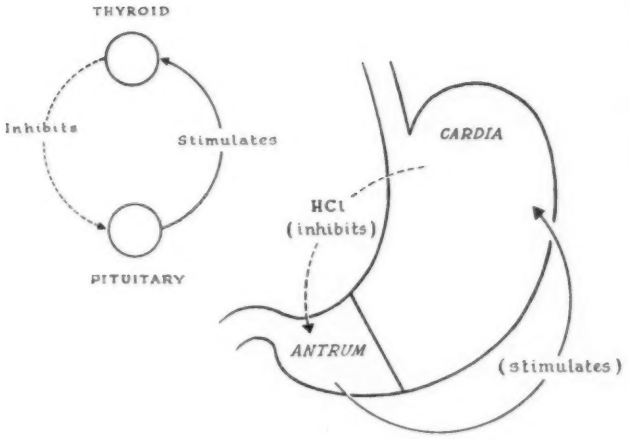
From the clinical standpoint, tumors of the thyroid appear to react as they do in laboratory animals; their growth may be stimulated by anything that depresses the function of the thyroid.

2) *Cancer of the stomach.*—About 70 percent of patients with cancer of the stomach have little or no free hydrochloric acid. It is unlikely that cancers inhibit the formation of acid and more likely that people with achlorhydria have an increased susceptibility to cancer of the stomach. Thus, cancer of the stomach is more common in persons with pernicious anemia (and achlorhydria) than in the population at large.

The antrum of the stomach is a known endocrine organ that elaborates a substance which stimulates the formation of acid by the cardia. Here perhaps is a direct axis, comparable to the thyroid-pituitary axis, in which primary failure of the target organ—in this case the acid-making cells of the cardia—results in a continuous stimulation of a compensatory mechanism and ultimately in the development of cancer. The antrum, in the absence of acid, is trying constantly to stimulate the cardia to make acid. Does achlorhydria result in prolonged stimulation of the antral cells and are the acid-making cells in the cardia constantly stimulated, in turn, by the hormone from the antrum? I have seen a patient with achlorhydria who had dozens of polyps on the mucosa of the upper two-thirds of the stomach but not a trace of a polyp on the antral mucosa. There is an analogy between this case and the development of adenomas of the thyroid in animals whose thyroid function is suppressed (text-fig. 3). There is also an analogy between the low incidence of cancer of the thyroid in the hyperfunctioning glands of patients with Graves' disease and the low incidence of cancer of the stomach in patients with gastric hyperacidity.

The gastric axis may not be so simple as the thyroid-pituitary axis, for the pancreas and other endocrine organs must be included in any study of gastric physiology. Zollinger and Ellison's (*13*) observations of the "ulcerogenic" islet-cell tumors of the pancreas that are associated with high output of hydrochloric acid and intractable duodenal and jejunal ulcers suggest that the pancreas may participate in the control of gastric acidity. The finding of adenomas of the pituitary, parathyroid, and adrenal cortex in these cases further suggests that several endocrine glands and, perhaps, even the hypothalamus may be involved. It would be interesting to know whether some of the remissions that have been reported following partial gastrectomy for incompletely removed gastric cancer were the result of removal of the source of antral hormone. It is interesting, also, to note that the prognosis of gastric cancer is much better in those cases in which acid is high, suggesting that this type of cancer is different from that associated with achlorhydria (*14*).

3) *Polyposis and cancer of the colon.*—Familial polyposis of the colon is a disease that is inherited as a Mendelian dominant. Polyps develop in



TEXT-FIGURE 3.—*Comparison Between the Thyroid-Pituitary Axis and the Axis That Controls Gastric Acidity.* If in the gastric cardia-antrum axis the hormone gastrin secreted by the antrum is analogous to thyrotrophin, a primary failure of the cardia to secrete hydrochloric acid might result in prolonged stimulation of the cardia by gastrin. Under such circumstances, persistent achlorhydria might result in the development of cancer of the stomach just as prolonged hypothyroidism in animals may result in cancer of the thyroid.

childhood, and inexorably go on to malignant change early in adult life. Is this type of cancer inherited as a genetic character, or is polyposis and its later malignant change the result of a hereditary deficiency in an enzymatic system and of a compensatory reaction to this deficiency? Perhaps in these colons there is a deficiency of some "signal substance" that controls the output of a "colonotrophic hormone." After years of exposure to the compensatory excess of "colonotrophic hormone," hyperplasias, adenomas, and finally cancers result. Perhaps administration of the specific chemical that is present in normal colons and deficient in patients with polyposis could prevent the development of polyps and cancers or even control the growth of some of the more differentiated cancers of the colon.[2]

4) *Cancer of the skin.*—When an area of the skin is injured or removed, there ensues a period of intense chemical activity designed to hasten the regrowth of the skin. Dunphy and Udapa (*15*) have shown by microchemical techniques that during this period chemicals necessary for repair are concentrated at the site of injury. But if the area to be healed is too large, it cannot heal. For months and years there are concentrated in this area all the trophic chemicals that promote the growth of skin. The failure of the target organ, *i.e.*, the skin, to do its job of covering the defect results in endogenous chemical stimulation of the cells around the defect. Finally, perhaps as a result of this stimulation, cancer develops.

[2] A case of familial polyposis has been reported in which the rectal polyps disappeared after the colon was removed. (Am. Surgeon 23: 577–586, 1957.)

Erosion and chronic infection, present so frequently in the cervices of parous women, could likewise evoke a chemical response designed to promote repair. Perhaps the compensatory reaction of healing ultimately results in cancer.

The lag period between the exposure of the skin to ionizing radiation and the development of cancer has never been clearly explained. If the effect of the radiation is genetic, one would expect the change in the genes to take place at once and the cancer to manifest itself promptly. Instead, there is no immediate carcinogenic effect, but over a period of years diffuse changes take place in the irradiated area and ultimately multifocal cancers develop.

The mechanism by which irradiation induces cancers of the skin may be similar to that by which, in animals, I^{131} suppresses the formation of thyroid hormone and induces cancer of the thyroid. The radiation may destroy the function of an area of skin and suppress its ability to elaborate a specific "signal substance." The body interprets the absence of the skin's "signal substance" in the irradiated area as the absence of skin, and it attempts to effect repair. The depressed function causes a compensatory oversecretion of growth-producing chemicals and results ultimately in the development of cancer.

Cancer of the skin following ingestion of arsenic or application of coal tars may be similar in etiology to the cancer of the thyroid that follows administration of thiouracil in animals. The tar or the arsenic may block the enzymatic system of the skin as thiouracil or iodine blocks the enzymatic system of the thyroid. "Hypodermism," a deficiency in the output of the "signal substance" of the skin, then results. This in turn causes a compensatory reaction that results in an excessive output of specific skin-growth factors and ultimately in the production of cancer.

B. Laboratory Evidence for the Presence of "Signal Substances"

From the laboratory, too, has come evidence suggesting that trophic substances affect the development of cancer even in organs that are not considered to be target organs or to have endocrine functions.

If a carcinogen is applied to a patch of a mouse's skin in quantities sufficient to evoke the development of cancer, and the carcinogen-treated epidermis is removed and transplanted to an untreated area before cancer develops, cancer develops not in the transplanted, carcinogen-treated graft, but in the site from which it was removed (*16*). Cancers that develop after implantation of a carcinogen in the muscle of normal rats can be prevented by hypophysectomy (*17*). Hypophysectomy also protects carcinogen-treated rats from developing hepatomas (*18*).

Furth (*19*) believes that the number of circulating leukocytes may be controlled by specific forces that stimulate or restrain their production and regulate their delivery, and he suggests that some of the chronic myeloid leukemias which he has observed in inbred strains of mice may have been conditioned neoplasms. Already it has been shown that injection of normal leukocytes will temporarily reduce the leukocyte

count of some patients with leukemia (*20*). In congestive splenomegaly, the leukocyte count tends to be low, perhaps, because a large number of normal functioning leukocytes are trapped in the enlarged spleen and the "signal substance" which they secrete depresses the output of a leukocytetrophic hormone.

There is mounting evidence that many tumors of the thymus and lymph tissue are dependent on hormones that have not yet been isolated. In animals thymic lymphomas can be induced by irradiation of the total body, but it is unlikely this result is a direct or mutagenic effect on the cells of the thymus. Local irradiation of the thymus alone does not cause malignant change (*21*); moreover, shielding of a single leg prevents the development of the thymic tumors. Similar protection is given by injecting normal bone marrow. Kaplan (*21*) suggests that " . . . the real carcinogenic agent is the normal endogenous growth stimulus for the thymus acting over a prolonged period upon the injured gland under circumstances in which it cannot respond normally."

The fact that mice are protected against malignancy by dissolving the carcinogen benzopyrene in mouse fat instead of in vegetable oil (*22*) suggests that there is a diffusible substance in the mouse fat that neutralized the effects of the benzopyrene or prevented the compensatory changes that otherwise cause cancer.

Trauma to skin, after the neoplastic phase is induced by application of a carcinogen, precipitates the formation of cancers that otherwise in the absence of further applications of the carcinogen would not develop (*23*). Growth of liver-tumor cells implanted in a healing wound has been reported to have been followed by regression of the neoplasm as the process of repair neared completion (*24*).

These observations and many others suggest that there are endogenous growth-stimulating carcinogens other than the trophic hormones made by recognized endocrine glands. Moreover, disturbances in the recognized endocrine system appear to produce changes in organs that are not ordinarily thought of as their target organs. Even the growth of tumors of the stomach and kidney seems to be related to the function of the endocrine glands. Implantation of estrogen pellets has produced tumors in the kidneys of female hamsters and nephrosis in the males (*25*). A fatal adenomatous lesion of the stomach has been produced in mice whose total body has been exposed to irradiation like that which produces tumors of the ovary and the pituitary (*26*). In rats, partial hepatectomy causes rapid growth of the liver of a parabiotic partner (*27*). Dried, frozen-skin grafts, whose cells are dead, have been shown to contain a substance that, when the graft is applied to the wound, prevents the contraction of scar tissue (*28*). These few isolated experiments and many similar ones indicate that the growth of normal cells, and perhaps also that of cancer cells, is controlled by complex chemical balances widely distributed throughout the body. Since many of these balances may be effected by a "contact" type of action like that of the organizers of embryonic development, it may be difficult to isolate the responsible substances.

VI. Development of Cancer in Atrophic Organs

If cancers always occurred in tissues that were hyperplastic and obviously stimulated by trophic hormones, it would be easy to accept hypersecretion of normal trophic hormones as the basic cause of cancer. But often cancers occur in organs that are atrophic, such as the senile prostate and the postmenopausal breast. These cancers may grow rapidly when hormones are given and may shrink or disappear when these hormones are withdrawn. This observation suggests that despite their origin in atrophic organs these cancers still depend on trophic hormones for their growth.

Although it seems strange that cancers should originate sometimes in the course of hyperplasia and sometimes in the course of atrophy, newer concepts of genetics and the concept of the latent or dormant cancer cell suggest an explanation of this paradox.

In Purves, Griesbach, and Kennedy's (*5*) experiment on induced thyroid cancers, a differentiated thyroid cancer was at first dependent for its survival on the presence of an excess of thyroid-stimulating hormone, but, later, after being transplanted through several generations of thiouracil-fed rats, it became undifferentiated and autonomous. This and many similar observations on tumors induced in the endocrine glands of animals suggest that the gene is not as stable as has been implied in the theories of both Mendel and Darwin. It appears that genetic changes in cells can be produced by alterations in the cells' chemical environment (*29*). Similar observations have been made in the study of insects and bacteria (*30*). Mutant strains of colon bacillus that show certain enzymatic peculiarities tend to revert to normal when the factors causing the mutation are withdrawn. It would be strange indeed if this were not the case, for if a gene is capable of exerting profound effects on the chemistry of the cell, it would be astonishing if the chemistry of the cell did not in turn affect to some degree the structure of the gene (*31*).

If genes are capable of reacting to their environment, if a high concentration of a certain chemical or the absence of such a chemical is capable of producing a minute reaction in the gene, then these induced changes will be inherited. If the same chemical environment persists, further minute changes in the genes will be produced and inherited. Ultimately this process may result in the production of significant changes in the cells. Foulds (*2*), in support of this view, has stated that "... the development of cancer is more like differentiation in reverse than like a mutation." In the differentiation of the embryo, organizing substances appear to exert a steady pressure toward differentiation along certain lines. In the development of cancer, trophic hormones appear to dedifferentiate the tissues and cause the reverse effect.

When subjected to stimulation by a trophic hormone, individual cells, or groups of cells descended from different individuals may respond in different ways. Thus asymmetrical enlargements of the thyroid may occur in response to overstimulation by thyrotrophic hormone. Adenomas or carcinomas may develop. Zondek and Leszynsky (*11*) have reported two patients with multinodular goiters whose nodules showed different

responses to changes in the output of thyrotrophic hormone. Some of the nodules disappeared when thyroid was fed and, when thyroid feeding was stopped, recurred in the order of their disappearance. Other nodules histologically similar to those that responded did not change in size. Apparently in this case small groups of cells and their descendants developed an unusual sensitivity to thyrotrophic hormone (*11*). Another example of the varying sensitivity of end-organ responses is seen in gynecomastia when a breast's increased susceptibility to a trophic stimulus may result in unilateral enlargement of the male breast.

If genetic alterations can be induced either by changes in the chemical environment or by "spontaneous" mutations, these alterations may give rise to a cell or group of cells that have a peculiar sensitivity to the chemical stimuli of growth. Even if the abnormal concentration of trophic hormones that evoked the mutation returns to normal, the mutant cells may remain hypersensitive to growth stimuli and may outgrow their normal neighbors. An unusual susceptibility of a single cell and its descendants to normal amounts of trophic hormones or a normal susceptibility of many cells and their descendants to excessive amounts of trophic hormones may be the basic cause of some of the abnormal growths that we call cancer.

Although modern genetics can suggest the process by which endocrine imbalances cause cells to become malignant and transmit the malignant change to their descendants, it is necessary, before the development of cancer in atrophic tissues can be explained, to introduce the concept of the latent or dormant cancer cell.

Many years ago Rous and Kidd (*6*) induced tumors by tarring rabbits' ears and showed that many of the tumors were conditional and dependent for their survival on continuation of the tarring. But tumors that regressed during intermissions of tarring recurred promptly at the same site when tarring was resumed. The application of a nonspecific irritant like turpentine, which by itself induced no tumors, evoked growths from previously tarred sites at the site of tumors that had regressed. Rous and Kidd believed that the cells of the tarred ears were in a subthreshold neoplastic state but were not capable in themselves of initiating tumor growth. According to this concept, the process of initiating the original tumor growth involved an irreversible change, for long after tarring had been discontinued and the resulting tumor had disappeared, the tumor could be evoked again by application of an irritating promoting agent. The changes of promotion, in contrast to those of initiation tend to be reversible. As soon as the stimulus that promotes the development of the tumor is withdrawn, most of the tumor cells revert to the subthreshold neoplastic state.

There appear to be any number of promoting agents, including chemical irritants, trauma, and heat. According to Deelman and van Erp (*32*), trauma applied after the pre-neoplastic stage usually stimulates the formation of neoplasms. MacKenzie and Rous (*23*) concluded that tarring a rabbit's skin makes many more epidermal cells neoplastic than

are apparent by forming tumors. They may be present in large numbers and persist for a considerable time after brief tarring yet give rise to no growths unless encouraged. The stimulus of normal healing will suffice to make some of them multiply and form tumors.

Once the changes of malignancy are initiated in the cell, they may either progress to frank cancer or they may remain latent. A cancer of the breast in a woman of 70 might have been initiated 30 years earlier when the breast was in an abnormally hyperplastic and stimulated phase. The cancer did not reach a clinical stage of development before the changes of the menopause reduced the concentration of an initiating agent. But the changes of neoplasia had nevertheless been induced, cells were permanently altered, and these cells were only waiting for some promoting agent to stimulate the development of clinical cancer. In conditioned cells the promoting agent that stimulates the tissues to develop clinical cancer may, as in experimental animals, be the result of local trauma, of irritation by some nonspecific promoting agent, or of infection with a virus.

VII. Possible Role of Viruses as Promoting Agents

Excessive stimulation by a trophic hormone stimulates a cell to divide but, before it divides, the cell must reproduce its normal quota of nucleoprotein. A stimulated cell's avidity for nucleoprotein may become so great that the cell not only reproduces its own nucleoproteins as fast as it can but also gobbles up any virus-like particles of nucleoprotein with which it comes in contact. These particles, incorporated into the chromatin of the cell, might disturb the cell's genetic balance. Thus exposure of a trophin-stimulated cell to a virus might hasten the development of the nuclear changes that lead to autonomy of growth. Viruses may be part of the material out of which cancers are built.

VIII. Suggestions for Investigation of the Role of the "Signal Substances" in the Production of Cancer

If we broaden our concept of the endocrine system to include all types of cells, we should search for the basic cause of cancer in the endogenous trophic substances that promote the growth of each type of cell. We should study the chemicals that cause differentiation and growth of embryonic tissues. We should experiment with tissue cultures in search of endogenous chemicals that specifically stimulate the growth of each type of cell. Furthermore, we should study the effects of known exogenous carcinogens, like coal-tar derivatives and ionizing radiation, to see whether these agents exert their effects directly on the cell or indirectly by blocking the formation of a "signal substance" and setting into motion a series of compensating reactions that cause overproduction of a trophic hormone.

In the concept of cancer that has been presented, the challenge to the investigator is to isolate the "self-identifying" or "signal substances" that the cells may make. Clinicians could then employ these substances, as we do the known hormones, to correct the chemical imbalances that lead to irreversible malignant change. Perhaps an extract of skin,

applied to an area that had been treated by a carcinogen or by intensive irradiation, could prevent the subsequent development of cancer.

If we could isolate the chemicals that act as "signal substances" for each type of cell, we might have a valid means of preventing the development of many cancers and of permanently controlling the growth of those that are still in the dependent phase.

Summary

1) In the endocrine system, stimulation of target organs by trophic hormones increases the rate of growth of the target organs' cells and causes hyperplasia and the development of adenomas and carcinomas.

2) In tissues other than those of the endocrine system there also is evidence to suggest that the growth of cells is dependent on specific trophic hormones.

3) It is possible that the growth of each type of cell in the body is stimulated by a specific trophic hormone made by some other type of cell.

4) A specific "self-identifying" chemical or "signal substance" made by each type of cell may control the output of the specific trophic hormone that stimulates the growth of that type of cell.

5) Hyposecretion of "signal substances" and compensatory hypersecretion of trophic hormones may be a basic cause of cancer.

6) There is evidence to suggest that genetic changes can be induced by alterations in the chemical environment of cells. Such changes induced by specific trophic hormones may be a major cause of cancer.

7) Cancer may result from stimulation of a normal cell by an abnormally high concentration of a trophic hormone or it may be caused by an abnormally active response of a single cell and its descendants to a normal amount of trophic hormone.

8) The secret of the abnormal growth that we call cancer may be revealed by further study of the factors that promote and control the growth of normal tissues.

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The Treatment of Spontaneous Tumors of the Dog and Cat with a Filtrate from a Tissue Lysate [1,2]

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A filtrate derived from mammalian tissue and bacterial growth, developed during the 10-year period 1945–55, has been used to treat a series of spontaneous benign and malignant neoplasms of dogs and cats. In a near-consecutive series of 23 cases, many tumors, including several that would ordinarily be expected to pursue a malignant course, have regressed completely for varying lengths of time. This report gives detailed clinical histories of these 23 cases supported in many instances by photographs of the tumors during the course of treatment. All cases are confirmed by tissue biopsy, and photomicrographs of biopsy material are included where pertinent.

The method of preparation for the material used in the treatment of these dog and cat neoplasms is described in detail.

While it is believed that the method of preparation and, presumably, the product obtained are unique in the experimental therapy of cancer, efforts to influence tumor growth with substances derived from homologous and heterologous tissues are not new. Among the numerous authors who have reported regressions of tumors in rodents after administration of tissue autolysates or extracts produced under conditions of retarded bacterial growth, several are particularly prominent (*1–6*). Chambers and Scott, on the other hand, have recorded a growth-stimulating effect when autolysates prepared under presumably sterile conditions were used in the treatment of Jensen rat sarcoma (*7*, *8*). The earlier of these two papers gives a review of the German literature on this subject. An excellent review of both growth-promoting and growth-inhibiting substances extracted from normal organs is included in a paper by Maisin and Pourbaix (*9*). Fichera has reported favorable results in the treatment of tumors with both autolysates and tissue extracts prepared in the presence of

[1] Received for publication May 20, 1957.

[2] This work has been supported, in part, by private funds from F. E. Scott, A. P. Miller, C. J. Ketcham, R. N. Echlund, M.D., J. R. Foust, M.D., R. B. Griffith, and J. L. Block. Animals with neoplasms were contributed by Drs. D. E. Jasper, J. B. Crundwell, J. H. Hensley, G. E. McClintock, and W. Shramm. The pressure chambers were designed and built by Boller and Chivens Engineering Company. Dr. B. G. Lamson reviewed the clinical and pathology material and assisted in the preparation of the manuscript. Valuable technical assistance was provided by Mrs. Beatrice Livingston and Mr. Dale Gillette.

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microorganisms (*10, 11*). No reports have been disclosed, however, of bacterial filtrates or autolysates prepared under conditions of prolonged incubation and increased pressure.

Evolution of the Process

This work was stimulated by the widespread speculation during the first quarter of this century that some product of tissue breakdown might possess growth-regulatory capacity. The first crude efforts in this laboratory, in 1945, consisted merely of enclosing various types of tissue, including human placenta, within mason jars at room temperature and allowing autolysis and fermentation to take place for about 10 days. The first tumor-bearing dog treated by extracts from this disintegrating tissue developed abscesses at the sites of injections, became very ill, and, in spite of antibiotic therapy, finally died. The tumor, a carcinoma of the mouth, sloughed away concurrently. Encouraged to continue by this undoubtedly fortuitous happenstance, the next year was spent in injecting dogs with clarified and sterilized filtrates from tissue-breakdown products that had a completely negative influence on tumor growth. In 1946 it was first observed that extracts of filtrates from decomposing tissue, fermented in adequately sealed mason jars which contained fermentation gases under greater than atmospheric pressure, were exerting some inhibiting effect on neoplastic growth. Metal, whipped-cream cans were then utilized for fermentation chambers in order to retain fermentation gases more effectively, with the final conclusion that tissue undergoing autolysis and fermentation under increased pressure yielded an extract that definitely warranted further investigation. Consequently, in 1948, a stainless steel pressure cell capable of withstanding preset pressures from 1 to 3,500 pounds per square inch was designed and constructed (figs. 1–3). With this device, tissue breakdown was studied under widely varying pressures by introducing compressed air or other gases into the pressure chamber. In 1949 a dog with spontaneous generalized lymphosarcoma was treated with a filtrate prepared in a container held at 25-pounds pressure with bacteria present during incubation. The dog's tumors clinically regressed, and is Case #1 of this report. The method of preparation of the filtrate in this case, with minor variations, was utilized in the series of cases in this report.

Methods

The process consists essentially of incubation of fresh mammalian tissue in the presence of microorganisms, at 25-pounds gauge pressure. Human placenta has been the major source of tissue though dog muscle was used in two cases of this series, and horse sarcoma was used in one.

Fresh human placentas are obtained with aseptic precautions from the delivery room, in a sterile metal container. If immediate pickup cannot be arranged, the placentas are stored in the refrigerator for several hours without apparent detrimental effect. No antibacterial preservatives are added.

The placenta is ground in a sterile meat grinder and 500 gm. placed in the 750 cc. capacity fermentation container. After cooling, 150 cc. of 0.9 percent sterile saline, previously boiled to remove oxygen, is added. In the majority of cases no bacterial cultures were added specifically but bacterial contamination from nonsterile placentas and from the air is inevitable. When bacterial growth has been prevented and the pressure-cell contents made sterile by the addition of 10.0 cc. of toluene, or 200,000 units of penicillin and 1.0 gm. of dihydrostreptomycin, the resulting filtrate does not possess tumor-inhibiting characteristics. When bacterial growth has been retarded, but not prevented, by the addition of either 2.0 cc. of toluene or 100,000 units of penicillin and 0.025 gm. of dihydrostreptomycin, active filtrates have still been obtained. Suppression of bacterial growth by these latter means was employed to slow the evolution of gases during fermentation, thus permitting more accurate manual regulation of pressure at 25 pounds and making fewer pressure adjustments necessary at night.

The top of the container is put into place and sealed. The two cylinders at the top of the container communicate with the inside of the pressure cell. These cylinders are equipped with plungers that can be raised or lowered by means of a screw thread to regulate internal pressure, which is registered on the gauge mounted on top. Also in the top of the container, a centrally located screw with ball-bearing seal communicates with the pressurized compartment (figs. 1 and 2). On some occasions completely anaerobic conditions were produced within the pressure cell but removal of the air trapped in the tops of the pressure-regulating cylinders does not appear to be an essential part of the process. The internal pressure in the container is regulated at 25-pounds gauge pressure by lowering the cylinder plungers with the plunger-screw adjustments. The entire pressure cell is then placed in a hot-air incubator at 40° C.

During incubation the internal pressure must be maintained constantly at 25 pounds. As bacterial fermentation commences with the evolution of gases, the screw plungers must be gradually raised to prevent the build-up of pressure above 25 pounds. At about 16 hours sufficient gas has usually evolved by fermentation so that the excess gas must be taken off from the center screw orifice and the 25-pounds pressure again regulated with the screw plungers. Pressure adjustments on these manually operated pressure cells have been made at least every half hour during the day and at least every 4 hours during the night. An incubation chamber with an automatic pressure-regulating system has recently been constructed.

Incubation is continued from 6 weeks to 3 months with maximum yield of active substance obtained at approximately 3 months. The container is then opened. The contents are extremely odoriferous, cream souplike in consistency, and have the color of port wine. After 3 months of incubation bacteria are still present, and many bizarre variants are observed. At 6 weeks a mixed flora of gram-positive and gram-negative cocci and bacilli are invariably present.

The *p*H ranges from 6.0 to 6.8. The semiliquid content is strained

through unbleached muslin previously washed to remove sizing. The liquid is passed through a single 25.0 cm. sheet of coarse filter paper (Aloe 42,700) three or four times until the filtrate is clear, and then is sterilized by filtration through a 100 cc. Seitz filter flask using type ST-3, size L-6 filter pads, with a porosity of 0.1μ. Approximately 450 cc. of filtrate is obtained, which is then drawn through sterile tubing into previously evacuated sterile rubber-capped bottles and is ready for use. The bottled material is stored in the cold room at 10° C. Initially it was suspected that a loss of tumor-inhibiting activity occurred after storage in the cold room for longer than 1 week at 10° C. Therefore, a large number of these pressurized containers were used in order to provide fresh material at weekly intervals for the treatment of the animal tumors of this study. Deviations from the method of preparation of the materials are noted in the individual case histories.

Bioassay and Toxicity Studies

The active principle of the filtrate has not been identified. Furthermore, no chemical assay of the product has yet yielded a dependable reference for standardization of dog and cat doses. The activity of various lots of filtrate has varied from one batch to another, and entirely empirical testing in the tumor-bearing host has been necessary to characterize the potency and dosage of each lot of material. The difficulties of developing a useful rodent bioassay for a tumor-inhibiting material, with a postulated stability of about 1 week, are obvious. It was therefore decided to use the filtrate directly on the larger animals with spontaneous tumors. It is now believed that the active material has reasonable stability beyond 1 week. This recent finding has made the development of a rodent bioassay with transplantable tumors possible. Results in this area will be the subject of a separate report (*12*). In spite of the lack of a standardized product, specific doses of crude liquid used in the treatment schedules in the individual case histories and toxicity studies are given in order to illustrate the dose range that has empirically been determined to have antitumor activity and to be nontoxic.

Toxicity studies have been performed in dogs and mice. There appears to be a striking difference between the tolerated dose in the normal animal and the response of the animal supporting a neoplastic growth.

Four dogs were given the filtrate in acute toxicity experiments. They received 1.0 cc. intravenously per pound of body weight every hour for 8 consecutive hourly injections. Animals were observed for 4 days for acute effects. Two normal dogs weighing 18 and 35 pounds, respectively, received a total of 144 cc. (18 cc. $\times$ 8) and 280 cc. (35 cc. $\times$ 8) intravenously in 8 hours. The animals continued to eat and showed no clinical signs of toxicity. The other two dogs each harbored malignant neoplasms. A German shepherd weighing approximately 55 pounds with a carcinoma of the breast and a 24-pound mongrel with lymphosarcoma were administered 440 cc. (55 cc. $\times$ 8) and 192 cc. (24 cc. $\times$ 8) of the filtrate, respectively. These tumor-bearing animals both developed bloody diarrhea,

vomiting, and a fever of 105° F. in approximately 18 hours after the first injection. Both died within 4 days. Autopsy revealed a congested, red, edematous mucous membrane of the large bowel without ulceration. Other viscera appeared grossly normal. No microscopic evaluation was attempted.

A large number of healthy adult mice have tolerated 2.0 cc. of filtrate intraperitoneally daily for many weeks. In contrast, mice with large transplantable tumors have died when given more than 1.0 cc. a day. If the transplanted tumors are small, 1.5 cc. may be administered without causing the death of the animals.

Selection of Case Histories

Following the first decisive tumor regression experienced during filtrate therapy (Case #1), reproducibility of the method of preparation of active filtrate was demonstrated by producing regression of a second malignant tumor (Case #2). It then became necessary to define as precisely as possible the range of physical conditions under which bacterial fermentation would yield a tumor-inhibiting substance. The procedure described under "Methods" was consequently varied by changing some of the many possible variables one at a time: The pressure during fermentation, temperature, length of incubation, buffers at varying *p*H, oxygen content, presence or absence of bacteria, type of bacteria present, and the nature of tissue constituent were explored. It became apparent that unbuffered bacterial fermentation of a tissue base controlled at 25-pounds gauge pressure and incubated at 40° C. under anaerobic or aerobic conditions, with or without specific seeding by known pure strains of microorganisms, yielded a material with tumor-inhibiting qualities.

During the period 1949–54, which was devoted to defining the proper conditions for fermentation just enumerated, a total of 48 dogs and 5 cats with spontaneous neoplasms were treated with a variety of filtrates. Of these 53 animals, 32 were treated with a filtrate prepared according to the specifications outlined. Adequate clinical records for evaluation were not available for 2 of these 32 animals. Three more animals have been excluded because slides of biopsy tissue were not available for permanent possession in this laboratory. And finally, four more have been excluded when review of biopsy slides failed to demonstrate an entirely convincing neoplasm. Of these 7 animals excluded for reasons related to evaluation of the histologic diagnoses, 4 experienced complete clinical tumor regression, 2 incomplete tumor regression, and 1 no effect on tumor growth.

The remaining 23 cases listed in chronological order in table 1 are reported in detail. All are supported by tissue diagnoses, with slides on file and available for review in this laboratory. Cases #18, 19, 20, and 22 were treated by D. E. Jasper, D. V. M., of the University of California at Davis and are recorded with his permission. All other animals were treated in North Hollywood by the author.

Estimates of tumor size in all animals are expressed in centimeters or inches, even though actual measurements were not made in every case.

TABLE 1.—*Chronological list of spontaneous dog and cat neoplasms treated with filtrate between July 1949 and January 1954*

Case	Date	Animal	Sex	Age (*years*)	Tumor	Length of treatment (*months*)	Clinical status at end of treatment	Follow-up after treatment	
								Duration (*months*)	Status
1	7/49	Mixed terrier...	M	3	Stem-cell lymphoma, generalized	2	Complete regression	1	Died of pneumonia. Tumor present.
2	10/49	Scottish terrier..	M	14	Melanoma of jaw....	6	Complete regression	15	Alive, free of tumor.
3	12/49	Wire-haired terrier	F	12	Adenocarcinoma, breast	3	Complete regression	8	Developed second cancer of breast, sacrificed.
4	1/50	Cocker spaniel..	F	14	Adenoma, possibly adenocarcinoma, of breast	4	Complete regression	18	Died, free of tumor.
5	1/50	Dalmatian.....	F	8	Squamous carcinoma of breast	7	50% regression..	0	Autopsy, residual tumor.
6	1/50	Collie.....	M	10	Adenocarcinoma, rectum	9	Complete regression	37	Autopsy, no tumor.
7	2/50	Cocker spaniel..	M	14	Adenoma, perirectal gland	4	75% regression..	0	Died during treatment.
8	2/50	Scottish terrier..	M	1	Mastocytoma.....	4	Complete regression	65	Alive, free of tumor.
9	4/50	Cocker spaniel..	M	13	Adenoma, perianal...	8	75% regression..	0	Sacrificed with tumor present.
10	4/50	Irish setter.....	M	6	Epidermoid carcinoma	3	Complete regression	25	Alive, free of tumor.
11	5/50	Scottish terrier..	F	7	Lymphosarcoma, generalized	1	Necrosis of tumor	0	Died during treatment.
12	5/50	Scottish terrier..	F	12	Mixed tumor, breast.	4	Complete regression	48	Autopsy, no tumor.
13	5/50	Mixed terrier...	M	?	Lymphosarcoma, generalized	1½	Necrosis of tumor	0	Died during treatment.
14	10/50	Long-haired yellow cat	M	4½	Mastocytoma.....	2½	Complete regression	18	Alive, free of tumor.
15	11/50	Siamese cat.....	F	8	Lymphosarcoma, cervical node	1½	Complete regression	29	Alive, free of tumor.

16	2/51	Boxer.....	M	7	Lymphosarcoma, generalized	1½ *Weeks*	Complete regression	2 *Weeks*	Died, after treatment.
17	7/51	Cocker spaniel..	F	14	Undifferentiated malignant tumor, breast	2½ *Months*	Complete regression	30 *Months*	Died, free of tumor.
18	12/52	Collie.....	F	9	Lymphosarcoma, generalized	41 *Days*	Partial regression	0	Died during treatment.
19	1/53	Red-and-white cocker	M	5	Lymphosarcoma, generalized	7	No effect.....	0	Died during treatment.
20	2/53	Buff cocker.....	F	6½	Lymphosarcoma, generalized	16 *Months*	No effect.....	0	Died during treatment.
21	3/53	Black cat.....	M	7	Lymphosarcoma, generalized	3½ *Days*	Complete regression	41	Autopsy, no tumor.
22	8/53	Black cocker....	M	12	Melanoma, jaw with node metastases	50 *Months*	Necrosis of tumor	0	Died during treatment.
23	1/54	Mixed black-and-white dog	M	10	Hemangiosarcoma...	1½	Complete regression	21	Died, free of tumor.

Dog and cat weights are all estimates based on the animals' breed, sex, age, and nutrition. The animals were not actually weighed, but estimated weights are included to provide some basis, if only approximate, for the recording of dose-weight relationships.

Case #1

This mixed terrier, weighing approximately 20 pounds, had a threefold enlargement of one tonsil which measured 3.5 × 1.5 cm. All superficial lymph nodes were enlarged, the suprascapular nodes measuring 5.0 × 3.5 cm. Liver and spleen were questionably enlarged to palpation. Biopsy revealed extensive proliferation of large reticulum-type cells and raised the question of lymphoma (fig. 4). The treatment was a filtrate prepared from horse sarcoma as a tissue base, 1.0 cc. given daily by intravenous injection. After 3 weeks of continued tumor growth, the intravenous dose was increased to 4.0 cc. twice daily. Eight days later noticeable tumor regression had occurred, but a temperature elevation of 105° F. with vomiting and dehydration also developed. After supportive therapy consisting of penicillin, cremosuxidine, and intravenous 1.0 percent saline, treatment was resumed 1 week later with smaller doses of 2.0 cc. 3 times daily. This was continued until 84 days of therapy were completed. By this time the lymph nodes, clinicially, had regressed completely. The dog developed pneumonia 1 month later and died; however, tumor regression persisted during this period. At autopsy only one enlarged 1.0 cm. suprascapular lymph node was found which histologically revealed lymphoblastoma (fig. 5). Grossly, the kidneys appeared normal but microscopic examination showed focal infiltration with immature lymphoid cells resembling those seen in the lymph node (fig. 6). Liver and spleen were grossly and microscopically normal.

Comment: Infiltration of a parenchymal organ, along with loss of lymph-node architecture in one suprascapular lymph node at autopsy, confirms the somewhat equivocal biopsy diagnosis of malignant lymphoma. Rapid tumor regression with resorption of breakdown tissue products is believed to explain the high fever, nausea, and vomiting experienced. Whereas the dog showed a complete clinical regression, residual neoplasm was present at autopsy. This observation suggested the desirability of continuation of therapy for a period after complete clinical regression is achieved. This procedure was followed in subsequent cases.

Case #2

Initial examination of this Scottish terrier revealed a swelling approximately 2½ inches long, 1 inch wide, and 1 inch thick in this 27-pound terrier's left mandible in the region of the canine and premolar teeth. The overlying mucosa was ulcerated, and the canine and premolar teeth were loose. Biopsy of the lesion revealed a malignant melanoma (fig. 7). There was no clinical evidence of dissemination of the neoplasm.

Daily injections of 1.0 cc. of filtrate were given intravenously for 7 days. During this time the lesion grew progressively larger, the canine tooth and the 3 premolar teeth sloughed out, and the mandible became very spongy. A week later the dosage was increased, 3 injections of 2.0

cc. being given intravenously each day at 4-hour intervals. The swelling of the jaw gradually subsided and healing of the overlying mucosal ulceration commenced. These injections were continued for a period of 30 days, at which time the tumor had regressed completely with total healing of the overlying ulceration. Thereafter, 1 day each week for a period of 20 weeks, the terrier was given 3 intravenous injections of 2.0 cc. of the filtrate. All treatment was discontinued after 6 months. The dog was last seen on July 4, 1951, 21 months after the diagnosis of melanoma was established histologically, when it was in good health and had no apparent tumor. Based on these clinical observations, regression of this neoplasm has persisted for 15 months after treatment was completed, without recurrence. No further follow-up was obtained.

Comment: This neoplasm was clearly malignant, histologically. There was no clinical evidence of metastasis but the lesion was locally aggressive. Local healing was complete and there was no recurrence for at least 15 months after cessation of therapy.

Case #3

This 18-pound terrier had nodular enlargements of all mammary glands on the right side. The lesion of the cephalad thoracic breast was discrete, measuring 3.0 × 2.0 cm. The caudal, abdominal, and inguinal breasts were involved by a confluent tumor mass measuring approximately 17.0 cm. in length and 5.0 cm. in width. None of the lesions were ulcerated. The tumor involving the 3 caudal breasts was removed surgically, but impending shock terminated the procedure before the cephalad discrete lesion could be removed. Histologic diagnosis was adenocarcinoma of the breast. Within 3 weeks nodular recurrences were apparent beneath the skin and along the skin incision. Biopsy revealed an infiltrating adenocarcinoma (fig. 8). A 3 months' intravenous course of therapy with the filtrate 1.5 cc. 3 times daily resulted in a slow, gradual, complete regression of the recurrent tumor beginning 3 weeks after commencement of treatment. The tumor of the cephalad breast shrank to a hard nodule. No recurrence was observed for 8 months when another tumor mass measuring approximately 8.0 × 6.0 cm. gradually developed in the left cephalad breast. The animal was sacrificed at the request of the owner because of its age. Autopsy revealed adenocarcinoma in the left cephalad breast. There was no gross tumor at the site of the previous surgery on the right and no internal metastases were observed. Histologic examination was limited to tissue from the new breast lesion, which revealed adenocarcinoma.

Comment: Biopsy here showed a relatively low-grade malignancy which was, however, definitely infiltrating. Whether the tumor arising 8 months later was a recurrence or new neoplasm is questionable. Autopsy revealed no gross metastases.

Case #4

This 14-year-old 32-pound female cocker presented a lobulated breast tumor involving the right inguinal breasts in a nonulcerating mass meas-

uring approximately 12.0 cm. in length and 7.0 cm. in width. There was no clinical evidence of dissemination. Biopsy was interpreted as adenoma of the breast, possibly low-grade adenocarcinoma (fig. 9). Treatment was initiated with 2.0 cc. of filtrate twice daily. The tumor increased in size during the first month, and the dose was increased to 2.5 cc. three times daily. Tumor regression then occurred slowly and gradually with complete regression achieved in 3½ months after onset of therapy. Treatment was continued for 12 additional days. Approximately 1½ years later, at age 15½ years, the animal died. Autopsy revealed a mitral vegetative endocarditis. At the site of the previous tumor only a 2.0 cm. hard lump was found. There was no other evidence of neoplasm. Gross metastases were absent. Histologic examination was not made.

Case #5

An ulcerated 4½- × 3-inch mass, 3 inches thick, occupied the region of one right inguinal breast in this 50-pound Dalmatian, and an external inguinal lymph node measuring 3 × 2 inches. Biopsy of the breast lesion showed squamous carcinoma extensively infiltrating normal breast structure (fig. 10). The lymph node was not biopsied. Intravenous filtrate was administered 10.0 cc. 3 times daily. The animal developed anorexia 17 days later and a temperature of 103° F. Treatment was discontinued for 3 days. Reinstitution of therapy, 6.0 cc. 3 times daily, for 38 additional days was achieved. The tumor gradually regressed to approximately 50 percent initial size by this date. The inguinal lymph node decreased in size until it was no longer palpable.

A month's therapy with similar doses of filtrate prepared with 200-pounds pressure was accompanied by growth of the tumor so that filtrate prepared at 25-pounds pressure, as detailed in the section entitled "Methods," was again used. Within 3 weeks the tumor had again regressed to one half initial size. Ten days later the tumor had regressed to about 5.0 cm. in diameter (fig. 11). The neoplasm measured 4.0 cm. in diameter and 1.0 cm. in thickness, 51 days later (fig. 12). Again the filtrate was changed to that prepared with a higher pressure of 100 pounds, and 6.0 cc. of the filtrate was given intravenously 3 times daily. Enlargement of the tumor occurred during 15 days of this therapy. The inguinal lymph node, however, never again became palpable. Poor veins necessitated cessation of therapy, and 12 days later the tumor measured 11.0 cm. in diameter and 4.0 cm. in thickness. The original solution prepared with 25-pounds pressure was then resumed intravenously, 6.0 cc. 3 times daily. This schedule was interrupted by death. Autopsy revealed coronary thrombosis. Residual tumor of the breast was present. The inguinal lymph node could not be found. There were no metastases grossly evident.

Comment: The cardinal necessity of preparing the filtrate under conditions of controlled pressure is illustrated by this case. Deviations from the optimum pressure of 25 pounds, on two occasions, resulted in filtrate preparations that were not associated with tumor regression. Return to the filtrate prepared according to the specifications outlined in this report was accompanied by tumor regression a second time. The final trial of filtrate therapy was interrupted by death from coronary thrombosis.

Case #6

A growth was observed protruding from the rectum of this 60-pound collie. By proctoscopic examination it extended for 10.0 cm. above the sphincter, was 2.0 cm. thick, and involved three fourths of the circumference of the rectal mucosa. The lesion was friable, bleeding readily during instrumentation. Biopsy of the lesion was diagnosed as adenocarcinoma (figs. 13 and 14).

Filtrate prepared from dog muscle with 2.0 cc. of toluene added at the onset of incubation was administered, 5.0 cc. 3 times daily at 4-hour intervals for a period of 36 days. Following diarrhea and vomiting the injections were discontinued for a week. Thereafter, injections were again given 3 times daily but the quantity was reduced to 4.0 cc. per injection. The growth appeared to have regressed completely within 65 days when the lesion was no longer palpable by digital examination. Treatment was continued, nevertheless, at intervals consisting of 3 daily injections for 4 consecutive days, twice a day during the next 2 months, and once a day the following 5 months. Treatment thus extended for a total period of 9 months, 7 months after clinical regression was complete.

The collie was examined every 6 months, including a proctoscopic examination, for 3 years after the termination of treatment and each time there was no evidence of recurrence of the tumor. The rectal mucosa remained smooth and healthy in appearance.

The animal finally died of hepatitis and nephritis at the age of 14 years. An autopsy was performed 44 months after regression of the tumor, and no gross evidence of rectal recurrence or metastasis was found. Histologic examination of rectum, liver, kidney, lymph nodes, bladder, and prostate were likewise negative for neoplasm. The appearance of the rectum, both grossly and microscopically, was normal.

Comment: The biopsy in this case was superficial and did not demonstrate invasion of submucosa or muscle. The lesion was not, however, a pedunculated growth that might conceivably have strangulated and been cast off. The involvement of the rectum was extensive, yet the tumor regressed without residual scarring of the rectum at autopsy. Presumably it involved only the mucosa and submucosa and had not invaded smooth muscle. Histologically the malignant appearance of the growth is clear cut. At no time was there evidence of metastases in this case.

Case #7

A 14-year-old cocker weighing 32 pounds presented an ulcerated perianal gland adenoma, 2 inches in diameter (fig. 15). Two months of intravenous filtrate therapy, 2.0 cc., 3 times daily, prepared with dog muscle and with 2.0 cc. toluene added, as in Case #6, was not accompanied by regression of this tumor. The dose was then increased to 5.0 cc. 3 times daily. A fever of 105° F. developed 14 days later, accompanied by diarrhea and vomiting. A 4-day rest period was permitted and then therapy resumed at 3.0 cc. 3 times daily. Thirty-seven days later the dog developed a bacteremia and died within 48 hours. By this time tumor regression to ½ inch in diameter had occurred. Autopsy again showed viable tumor without histologic evidence of the regression.

Comment: This benign tumor regressed slowly (by 75%) during treatment. Histologic evaluation revealed no evidence of necrosis or tissue disintegration during this slow subsidence.

Case #8

A tumor 1.5 cm. in diameter with the histologic characteristics of mastocytoma was removed surgically from the lateral surface of the right hind leg of this 24-pound Scottish terrier. The tumor recurred and again was removed surgically 3½ months later. Microscopic examination again revealed mastocytoma. The tumor reappeared 45 days later, together with 18 additional tumors involving various areas of the skin over all parts of the body. These ranged in size from 0.5 cm. to 2.0 cm. The new tumors included 2 in each of the following areas: the tongue, the medial surface of the left hind leg, the lateral surface of the left hind leg, and the lateral surface of the right hind leg; 5 tumors on the testicles, 1 tumor on the left side of the thorax, and 1 on the right upper lip. The appearance of the testicular lesions and a representative lesion on the skin of the thorax are shown in figures 16 and 17. Biopsies were taken from several tumors for histologic examination, all of which again had the appearance of mastocytoma (figs. 18 and 19).

For 28 days the dog was given injections of 2.0 cc. of the filtrate intravenously 3 times daily at 4-hour intervals. Within 5 days, evidence of tumor regression was observed. The considerable regression which had occurred by 9 days is shown in figure 20.

Treatment was discontinued after 28 days because the dog developed diarrhea, vomiting, and a temperature of 104.3° F. Although treatment was resumed 5 days later, it was stopped again after 2 weeks because the dog again developed a temperature of 105.2° F., diarrhea, and vomiting. The progressive regression of the tumors on the dog's testicles and skin at 33 and 45 days is shown in figures 21, 22, 23, and 24. Treatment was reinstituted after a 5-day rest period and biopsies of several of the tumors were again prepared for histologic examination. Clinically the tumors appeared to have regressed approximately 90 percent at this time. While the laboratory classified the tissue sections as mastocytoma (fig. 25), the tumor cells contained less chromatin, the nuclei were smaller, and there was a decrease in cellularity.

By 60 days no visible tumor remained. Normal growth of hair returned along with normal skin pigmentation. Figures 26 and 27 show the appearance of the testicles and previously illustrated skin lesions at this time. A section of skin from the previous site of one of the skin tumors on the dog's thorax was examined histologically. As shown in figure 28, only subcutaneous fibrosis was seen. Treatment was discontinued just short of 4 months after initial therapy.

When this Scottish terrier was last examined on April 15, 1956, no evidence of tumor existed. Thus, complete regression of the tumors appeared to have been achieved for at least 5 years and 5 months.

Comment: Toluidine blue and Giemsa stains both failed to demonstrate specific metachromatic cytoplasmic granules in this tumor. These specific granules which are

diagnostic of mast-cell tumors are often rare or absent in the more pleomorphic neoplasms. The initial involvement of the skin of the testes in this case is, in addition, characteristic of mast-cell tumors. Not all observers agree that mast-cell tumors are invariably malignant neoplasms. However, experience of veterinarians generally supports the view that a mast-cell tumor which has twice recurred and has then become generalized, as occurred in this case, can be expected to pursue a malignant course.

Case #9

This 34-pound dog with a 3.5 cm. perianal adenoma (fig. 29) was used to determine whether tumor sensitivity to the filtrate decreased following repeated courses of therapy. Treatment was administered, 2.0 cc. 3 times daily, for 55 days until the tumor was approximately 50 percent of initial size, and then discontinued. One month without therapy was accompanied by regrowth of the tumor to the original size. The appearance of the neoplasm at this time is shown in figure 30. Seventy-five percent regression of the tumor again occurred when the same dose schedule was reinstituted for 2½ months, followed again by growth over a period of 45 days to slightly larger than initial size when treatment was discontinued. This procedure was performed 4 times. Both regressions and regrowth occurred more rapidly as the experiment progressed. Further treatment became impracticable because of poor veins, and the dog was sacrificed with the tumor still present.

Comment: This benign tumor retained its capacity for regrowth and its histologic identity following four separate partial regressions associated with four courses of filtrate treatment. Decreased tumor sensitivity to the effect of the filtrate did not appear following multiple treatments.

Case #10

A tumor of uncertain type was first removed surgically from the skin of the scrotum of this 60-pound Irish setter. The wound had opened up and filled in with exuberant granulation tissue 17 days later. The margins of the defect were raised and firm. A biopsy of this margin was diagnosed as epidermoid carcinoma (fig. 31).

Figure 32 shows the external characteristics of the lesion prior to treatment, which was commenced with 6.5 cc. of the filtrate intravenously, 3 times daily at 4-hour intervals. These injections were discontinued after 14 days when the dog developed diarrhea, vomiting, and a temperature of 105° F. Supportive treatment was then given for 10 days. Injections of the filtrate were resumed but the dose was reduced to 4.0 cc. per injection, 3 times daily. Figure 33 shows the partial regression 38 days after the onset of treatment. When healing was 95 percent complete, a second biopsy of the affected area was taken, which showed normal epidermoid inclusions but no neoplastic cells or tumor growth. Although the lesion appeared to have completely regressed 45 days later, treatment was continued for another 2 weeks.

This Irish setter was last seen on August 1, 1952. At that date there were no signs of the tumor, which was 25 months following its regression during treatment.

Comment: The initial biopsy in this case was interpreted as squamous-cell carcinoma. The neoplastic epithelium was well differentiated and the difficulty of distinguishing squamous carcinoma of low grade from pseudoepitheliomatous hyperplasia about the borders of a benign ulcer was recognized. Although the case was diagnosed as cancer, the diagnosis was admittedly not certain beyond all doubt.

Case #11

All palpable lymph nodes of this 24-pound Scotty were enlarged, the prescapular nodes measuring 2 inches in diameter. Liver and spleen were enlarged to palpation. Biopsy revealed lymphosarcoma (fig. 34). Treatment was commenced with 3.0 cc. 3 times daily. No effect was obtained during the first 3 weeks of therapy. The liver and spleen returned to normal size followed by rapid node regression proceeding to complete liquefaction, with a 75 percent decrease in size of nodes occurring within 3 days. An elevation to 106° F. with diarrhea preceded this softening of neoplastic tissue by 48 hours. The tumor continued to regress until the animal's death 4 weeks after onset of therapy and 1 week after the first decrease in tumor size was observed. An autopsy was performed. Liver and spleen were grossly normal. The involved lymph nodes appeared as liquefied necrotic masses of tissue approximately 10 percent of their original size. Microscopic examination of tissue was not performed.

Comment: This case again illustrates the danger to the host of too rapid breakdown of tumor tissue. Lymphosarcoma in the dog appears to be particularly prone to rapid involution with necrosis. The earliest effect of the filtrate in dogs with lymphosarcoma has been shrinkage of the liver and spleen. Possibly this may serve as a warning to withhold or reduce the filtrate dose.

Case #12

This 27-pound Scottish terrier had a benign, ulcerated, firm mixed tumor of the right cephalad-inguinal breast measuring 8.0 × 4.0 cm. (fig. 35). She was initially treated with filtrate prepared under sterile conditions with penicillin (200,000 units) and dihydrostreptomycin (1.0 gm.) added to the pressure chamber. She received 4.0 cc. 3 times daily for 17 days, during which period the tumor doubled in size. This filtrate was discontinued and treatment commenced with a bacterial filtrate prepared as described under the section entitled "Methods," dosage 2.0 cc. 3 times daily. Tumor growth slowed immediately and within 3 weeks began to regress. There were no signs of toxicity or temperature elevation. The tumor disappeared within 90 days after inauguration of the second filtrate therapy. Treatment was discontinued 19 days later. This animal was observed for 4 years and had no recurrence. Autopsy was performed following death at age 16, with no tumor demonstrable.

Comment; Filtrate prepared with sterilizing quantities of penicillin and streptomycin had no tumor-inhibiting effect on this benign neoplasm during a 17-day trial. When filtrate, prepared with bacteria present during incubation, was used, as detailed in this report, the regression occurred.

Case #13

All the palpable lymph nodes of this 60-pound mongrel were enlarged, along with splenomegaly and hepatomegaly. The anterior cervical nodes

measured 10.0 × 8.5 cm., the prescapulars 7.0 × 6.0 cm., and the popliteals 3.5 × 6.0 cm. By X-ray examination it was determined that mediastinal nodes were also enlarged. The dog was in respiratory distress and appeared in an advanced stage of malignant lymphoma. Biopsy of a superficial lymph node revealed lymphosarcoma (fig. 36). Treatment was administered over a period of 42 days. After receiving 5.0 cc. 3 times a day for 14 days, a fever of 104° F. developed which necessitated interruption of the treatment for 4 days. Tumor regression was apparent by this time. Five days later a second elevation to 104° F. occurred and treatment was again stopped. Following another 5-day rest period, the temperature became normal and treatment was resumed. By this time the tumor had regressed approximately 50 percent. Another 10 days of therapy resulted in a third febrile episode. Fever (106.5° F.) this time was accompanied by bloody diarrhea. Both persisted until the death of the animal 6 days later. The tumor had regressed approximately 75 percent at this time. Autopsy showed liquefaction of tumor tissue. Involved lymph nodes were largely replaced by a creamy-yellow, purulent-appearing exudate.

Comment: Partial regression occurred but the dog died during treatment. Therapy in such an advanced stage of disease is hazardous. Tumor regression accompanied by high fever and bloody diarrhea occasionally forewarns a fatal outcome.

Case #14

This 8-pound male cat had a lesion 1.5 cm. long and 1.0 cm. wide on its upper-right eyelid (fig. 37). There was no clinical evidence of dissemination. The cat was given 3.0 cc. of the filtrate intravenously 3 times a day at 4-hour intervals. The lesion continued to grow for about 2 weeks when it began to show evidence of softening, as is shown in figure 38. A biopsy of the lesion made at this time was diagnosed as mastocytoma (fig. 39). Cytoplasmic granules in this tumor are shown in figure 40.

Daily injections of the filtrate were continued without interruption since the cat at no time showed a toxic reaction to the treatment. As seen in figure 41, the tumor appeared to have regressed considerably by January 4, 1951, after 67 days of therapy. Two weeks later, when treatment was discontinued, the tumor appeared completely regressed as is evident in figure 42.

There was no evidence of regrowth of the tumor on August 16, 1952, when the cat was last seen. Thus, the tumor apparently had completely regressed for a period of at least 18 months.

Comment: Demonstration of mast-cell granules by Giemsa stain established the identity of this tumor. Mast-cell tumors are not viewed as invariably malignant neoplasms by some observers. There was no evidence of dissemination in this case.

Case #15

Swelling of the anterior cervical lymph nodes was first observed in this 7-pound Siamese cat on November 3, 1950. The swelling continued to

grow larger during a course of antibiotic therapy until December 27, 1950, when it was approximately 6.0 cm. in diameter, as shown in figure 43. A biopsy of the lesion revealed lymphosarcoma (figs. 44 and 45). There was, however, no evidence of generalized lymphadenopathy, splenomegaly, or hepatomegaly.

Daily injections of 4.0 cc. of the filtrate were given intravenously at 3-hour intervals. On this day the tumor became fluctuant and the cat became extremely ill, registering a temperature of 105° F., with diarrhea and vomiting. The tumor area was drained of a semiliquid material; injections of the filtrate were discontinued, and supportive treatment was given.

Treatment was resumed after 6 days; however, the dosage was reduced to 1.0 cc. of the filtrate, given 3 times a day at 4-hour intervals. The lesion appeared to have healed completely 1 month later when all treatment was stopped. No signs of the tumor were thereafter observed. The cat was last examined on July 9, 1953, a period of 29 months after the tumor had completely regressed. No discernible signs of the tumor were detected during this examination.

Comment: Biopsy of the 6.0 cm. lymph node revealed lymphosarcoma. There was, however, no evidence of disseminated malignant lymphoma. It is recognized that bizarre hyperplasias of lymph nodes may occasionally mimic histologically a malignant lymph-node disease. The diagnosis here is not entirely secure but the very large size and nonfluctuant nature of the neck mass which did not respond to antibiotics favors the interpretation given.

Case #16

Within a 3-week period this 40-pound boxer had rapidly and progressively developed approximately 200 subcutaneous lesions that were both visible and palpable as firm nodules ranging in size up to 3.5 cm. in the skin of his body (fig. 46). Between the larger lesions visible in the photograph, many minute nodules were widely dispersed in the skin. Liver and spleen were both enlarged to palpation. Peripheral nodes were not palpable. Biopsy of several skin lesions were diagnosed as lymphosarcoma (fig. 47).

For a period of 15 days, the dog was given four 5.0 cc. intravenous injections daily at 3-hour intervals. Filtrate prepared with 2.0 cc. of toluene added was used. Tumor regression commenced 7 days following therapy. Figure 48 shows the decline in prominence of a skin lesion during this period. When the boxer developed a temperature of 104.2° F., with vomiting and diarrhea, treatment was discontinued and supportive treatment was administered for the next 4 days.

Reduced injections of the filtrate were then resumed, 3.0 cc. 3 times a day at 4-hour intervals, for a period of 10 days. Treatment was again discontinued because of the development of symptoms of toxemia. After a 3-day rest a final 10 days of therapy were given. The tumors appeared to have regressed completely by that time, March 17, 1951 (fig. 49), 43 days after therapy with filtrate was commenced. Hair grew back in the former sites of the skin nodules. These areas were only identified by

the small amount of residual pigmentation deposited in the otherwise white skin of the dog.

The boxer contracted pneumonia on March 26, 1951, and died 5 days later. An autopsy was performed. Sections of the skin where the tumors had been were examined histologically. Small foci of cellular infiltrations were still present. The cells of this infiltration, however, appeared to be more characteristic of inflammatory cells than neoplastic lymphocytes, as can be seen from figure 50. Liver and spleen were grossly normal and lymph nodes were not enlarged. No other histologic examination was performed.

Comment: The malignant nature of this neoplasm was established. Follow-up was unfortunately prevented by pneumonia, so the possibility of recurrence at a later date remains unanswered.

Case #17

A rapidly growing tumor measuring 13.0 × 10.0 cm. was removed from the breast of this 34-pound cocker spaniel. After 14 days a recurrence appeared at the site of the surgery, reaching the size shown in figure 51 in about 6 weeks. A biopsy of this recurrence was diagnosed as undifferentiated malignant tumor, possibly amelanotic melanoma (fig. 53).

Three daily intravenous injections of 2.0 cc. of the filtrate at 4-hour intervals were commenced 10 days later. Filtrate prepared with 2.0 cc. of toluene was used. Symptoms of toxemia were present but not to a degree that necessitated cessation of therapy. The dog's tumor regressed rapidly. The only remaining evidence of the tumor 20 days after treatment was started was a 2.5 cm. hard nodule immediately surrounding one of the cocker spaniel's nipples. Treatment was continued for another 52 days, but this hard nodule did not disappear. The appearance of this nodule a week after cessation of therapy is shown in figure 52. The dog was examined every 6 months following termination of therapy. No evidence was found in these examinations of tumor regrowth, though the hard breast nodule persisted The cocker spaniel died of nephritis on March 19, 1954, 30 months after termination of therapy. An autopsy yielded no gross evidence of tumor other than the hard persistent nodule in the breast area. Sections of tissue from the breast area where the tumor had been located, as well as from the principal organs of the body, were examined histologically. No evidence of neoplasm was found. The persistent hard nodule in the breast region proved to be a mixed tumor (fig. 54). In the area of the original malignant tumor only scarring and a few deposits of melanin pigment were observed (fig. 55). It is concluded, therefore, that this cocker spaniel experienced a complete tumor regression for 30 months.

Comment: The exact histogenesis of the original tumor is uncertain, but its malignant nature was clear from its undifferentiated appearance and its infiltrating method of growth. The finding at autopsy of deposits of melanin pigment within scar tissue at the site of the original tumor suggests that it may have been a relatively amelanotic melanoma. The hard nodule proved to be composed largely of stromal tissue with cartilaginous metaplasia which was interpreted as a mixed tumor of the breast.

Case #18

All external lymph nodes of this collie were enlarged, the anterior cervical and prescapular nodes measuring 7.5 cm. in diameter. Biopsy of a precrural node revealed lymphosarcoma. Treatment with filtrate prepared with 2.0 cc. of toluene added, 10.0 cc. 3 times daily for 9 days, was well tolerated. During the next 3 days slight regression in size of nodes was accompanied by slight fever and depression. The filtrate dosage was reduced; but fever and anorexia persisted over the next 3 days. Lymph-node regression was definite. On the 16th day, fever of 105.4° F. and diarrhea developed. Treatment was discontinued. Marked reduction in size of all lymph nodes had now occurred, with the exception of the left prescapular which appeared involved in an inflammatory process. Penicillin was given. Two days later the prescapular area was drained of 30.0 cc. of creamy-yellow exudate. Temperature slowly subsided and on the 20th day treatment was resumed with filtrate, 3.0 cc. 3 times daily. At this time most nodes had increased slightly in size since the 16th day. Over the 2-day period the filtrate dose was increased to 10.0 cc. 3 times daily. Lymph nodes continued to grow and now approached preregression size. Six days later fever of 104.5° F. returned; nodes became softer without decrease in size, and the filtrate dosage was variably decreased depending on the dog's condition. Fever persisted over the next 2 weeks as the lymph nodes slowly regressed for the second time.

The course remained febrile; the dog was unable to eat and finally died 41 days after inauguration of therapy. Autopsy revealed lymphosarcoma involving the spleen and multiple lymph nodes. Histologic evaluation of the lymph nodes showed much edema and hemorrhage, and persistent invasion of the capsular tissue with lymphoid elements (fig. 56).

Case #19

All external lymph nodes of this cocker spaniel were enlarged, the prescapular nodes to 3 inches in length. The tonsils were greatly enlarged. The iris of each eye and the left upper lip also appeared infiltrated with tumor. Severe exertional dyspnea was present. Biopsy of a superficial node showed lymphosarcoma (fig. 57).

Filtrate prepared with penicillin (100,000 units) and dihydrostreptomycin (0.025 gm.) was used, treatment consisting of 4.0 cc. 3 times daily. In 3 days an elevation to 105.5° F. occurred with depression and diarrhea. Dosage was reduced to 1.0 cc. 3 times a day, but treatment was continued. The temperature remained elevated to 104° F. for 3 more days until the death of the animal. Autopsy revealed generalized lymphosarcoma. No gross effect of the filtrate upon the tumor was observed.

Comment: Animals near death from cancer are not likely to survive the reactions associated with tumor effect.

Case #20

The prescapular lymph nodes of this cocker spaniel measured 7.5 × 5.0 × 2.5 cm. Popliteal nodes measured 2.5 × 2.5 cm. Cervical nodes

were also enlarged. Biopsy of a popliteal node showed lymphosarcoma (fig. 58). Treatment consisted of filtrate prepared with penicillin and streptomycin, as in Case #19, 2.5 cc. 3 times daily through a polyethylene catheter in the right jugular vein, for 17 consecutive days until death. Tumor regression was not observed. Fever appeared on the 11th day of therapy and an abscess was drained at the site of biopsy. Fever continued and the dog's clinical condition declined. Infected thrombus about the polyethylene catheter probably contributed to the demise. Autopsy revealed twofold and threefold enlargement of all lymph nodes. Histologically these showed lymphosarcoma, hyperemia, and hemorrhage. A vegetative endocarditis undoubtedly contributed to the animal's death.

Case #21

On March 21, 1953, this small 4½-pound cat developed severe icterus and massive liver enlargement which filled three quarters of the abdomen. All palpable lymph nodes were greatly enlarged up to 5.0 or 6.0 cm. as shown in figure 59. A biopsy of a popliteal lymph node was reported as lymphosarcoma (fig. 60).

Intravenous injections of 1.5 cc. of the filtrate, prepared as in Case #19, were given to the cat 3 times daily at 4-hour intervals, starting March 21, 1953. Extreme symptoms of toxemia were apparent 3 times during the course of treatment. Each time these symptoms developed, treatment was discontinued and supportive treatment was given for 4 or 5 days. Blood transfusions also were given at these times. The reactions were so severe in each instance that the cat was not expected to recover. About 7 days after each reaction, however, the cat regained strength and treatment was resumed. Lymph nodes and liver gradually receded. All tumors appeared to have completely regressed by July 3, 1953, and treatment was stopped after 100 days of therapy. The regression of the cat's enlarged cervical lymph nodes can be observed by comparing the photographs in figures 59 and 61 taken on July 3, 1953.

This cat lived in the laboratory in apparent good health until death from liver and renal disease in December, 1956. Hence, regression appeared complete for a period of 41 months. An autopsy was performed. There was no gross evidence of neoplasm. The liver was slightly smaller than normal and focally fibrotic. Lymph nodes were normal in size. Microscopic examination revealed extensive portal scarring in the liver (fig. 62) but no evidence of lymphoma in lymph nodes or in other organs.

Comment: According to any usual clinical and histological criteria this cat had a malignant lymphoma. The animal's freedom from disease of the lymphoid system, at autopsy 41 months after regression, is documented by autopsy microscopic examination.

Case #22

This cocker spaniel had a pink fungating growth on the left side of the hard and soft palate. It measured 1½ inches in diameter and was raised above the surface mucosa. Regional lymph nodes in the parotid area on the left were stony hard and measured up to 2 inches in diameter. Biopsy

showed melanoma (fig. 63). For 33 days 5.0 cc. of filtrate prepared as in Case #19 was administered 3 times daily. Temperature elevation occurred after 22 days and bloody diarrhea after 30 days. A 3-day rest period was followed by renewed therapy at the high dose of 10.0 cc. 3 times daily with a new supply of filtrate. Fever to 106.2° F. developed on the 2nd day of this new regime. The oral tumor became black and necrotic in appearance. On the 5th day the tumor appeared about ready to slough away and the lymph-node areas became fluctuant and were drained surgically. Therapy with filtrate and supportive measures including intraperitoneal infusions continued for 9 more days when the animal died. At that time the tumor had sloughed to the level of the palate surface. The areas appeared pink and viable. At autopsy the decrease in size of the oral tumor was confirmed. The involved lymph nodes of the neck showed liquefied centers and confirming margins of granulation tissue. Death was apparently caused by a perinephric abscess complicating an intraperitoneal infusion administered 9 days before.

Comment: This animal was doing well when a mishap during intraperitoneal infusion for supportive purposes eventually led to the development of a perinephric abscess. Prior to this accident the primary tumor had decreased in size and partially sloughed away. Lymph nodes presumably containing metastatic tumor had become fluctuant.

Case #23

In January, 1954, examination of this dog revealed multiple nodules in the abdominal skin. The largest of these measured 3.0 cm. in diameter and appeared on the ventral surface of the abdomen near the penis as shown in figure 64. Palpation of the spleen indicated massive enlargement of this organ. A tissue section from the skin lesion was diagnosed as hemangiosarcoma (figs. 65 and 66). Treatment was commenced February 1, 1954, with intravenous injections of filtrate prepared as in Case #19, 2.0 cc. 3 times a day at 4-hour intervals. This schedule was well tolerated for 13 days when the dog showed symptoms of toxemia, and injections were discontinued for 3 days. A repeat biopsy showed viable tumor with some hemorrhagic necrosis and influx of inflammatory cells (fig. 67). Surgical exploration of the abdomen was performed in order to evaluate the splenic mass which had by this time regressed in size by approximately 50 percent. A dark-red, hemorrhagic-appearing, mass, the size of a tennis ball, occupied the central portion of the spleen. This mass was adherent to the surrounding omentum. Lymph nodes near the hilum of the spleen measured up to 2.5 cm. in size and were dark red and hemorrhagic in appearance. No biopsy was taken. Treatment was resumed and a gradual regression of the skin lesions and the palpable splenic mass occurred over the next 18 days. Figures 68, 69, and 70 illustrate the decrease in prominence and size of a skin lesion during this period. On March 6, 1954, the site of the skin tumor was again biopsied. No clear-cut tumor was observed, the appearance being more that of vascular scar tissue with inflammatory cell influx (fig. 71). The spleen was still palpable at this time so treatment was continued for another 13

days. At the end of this period there was no clinical evidence of disease.

The dog was unfortunately killed by an automobile 21 months later. Autopsy was performed. There was no gross evidence of tumor. The spleen was normal in size and contained a hard, fibrous, circumscribed scar 2.5 cm. in diameter. Hilar splenic nodes were normal in size. Microscopic examinations were not performed.

Discussion

The nature of the active principle of the filtrate described in this report is not known. It appears that microorganisms are necessary for the production of the active filtrate. Admittedly, it has not been conclusively established that microorganisms are essential to the process, though the exclusion of all organisms from the fermentation chambers by the use of toluene or penicillin and dihydrostreptomycin in appropriate concentration is associated with apparent loss of tumor-inhibiting properties. At any rate, dog and cat tumors treated with filtrate prepared under sterile conditions have shown no tendency to regress. In several cases an actual acceleration of tumor growth has been suggested when the sterile containers have been incubated anaerobically. Similar growth-accelerating results have been reported by Chambers and Scott (*7*, *8*), as already mentioned. Obviously, more information is needed based upon detailed bacteriologic studies, both during and at the conclusion of the fermentation process. Such studies are now in progress.

Initially it was thought that anaerobic fermentation was essential to the process and many of the animals in this series were treated with filtrate prepared anaerobically. It is now believed that the elimination of the air trapped in the upper portions of the screw cylinders and the boiling of saline for removal of oxygen are not necessary for satisfactory preparation of the active material. Both aerobic and anaerobic methods of filtrate preparation were used in this series of cases without noticeably related variation in potency.

The function of increased pressure in the process has been extensively investigated. Active filtrates are not obtained unless the pressure is held at 25 pounds per square-inch gauge pressure. That pressure has an influence on the chemical changes occurring within the chamber is shown by the variation in color of the final product. At 15-pounds pressure the resultant filtrate will be nearly clear with only a faint-red color. At 25-pounds pressure and above the filtrate is a deep port-wine color.

Three months' incubation at 40° C. has been selected after varying the time of incubation from 2 weeks to 1 year. There is noticeable tumor-inhibiting activity at 2 weeks and approximately 75 percent of ultimate activity at 6 weeks. Incubations longer than 3 months up to 1 year have not noticeably increased or decreased the potency of the product.

Incubation temperatures of 60° C. or above, which inhibit bacterial growth, have not been associated with active filtrates. Temperatures lower than 40° C. appear to require a longer period to develop a comparable activity.

Human placenta was used as a tissue ingredient in the majority of preparations. The first case in which clinical regression was experienced, however, was treated with a filtrate prepared from horse sarcoma. Accumulated experience with the method now suggests that any animal tissue source is satisfactory, though only dog muscle, horse sarcoma, and human placenta were used in the preparation of materials in the present series. A meat-extract base has been substituted for fresh mammalian organ tissue and the results thus far have not been impressive.

The possible relationship of the present work to Coley's toxins and Shear's polysaccharide warrants comment. A historical review of Coley's efforts, which includes an account of the various methods of preparation of toxins, has been contributed by members of Coley's family (*13*). There is no indication that either prolonged incubation or increased pressure was considered an essential part of his process. On the other hand, one might speculate that an occasional bacterial culture in Coley's hands may have been incubated in closed containers, which allowed pressure to develop. It probably can never be established as fact, but the idea that the active material reported here is similar to that which produced the rare but convincing regressions of human cancer treated with Coley's toxins is an interesting one.

Shear's summary of his own work mentions the increased sensitivity of tumor-bearing animals to his necrotizing polysaccharide when compared with normal controls (*14*). To this extent the tumor-inhibiting agent described here is similar to Shear's material. However, several important differences in clinical response to the two materials are evident from the case histories presented. In the present report tumor regression is not invariably accompanied by necrosis; necrosis when present does not suggest a vascular injury with hemorrhage and central distribution, and there has been no demonstrable loss of tumor-inhibiting potency with multiple courses of therapy. Furthermore, with filtrate described in this report, tumor response is often delayed, or at least slow, and does not occur in a few hours after a single injection, as described in the use of Shear's material. For these reasons it is believed highly improbable that the active fraction of the material reported here is identical to Shear's polysaccharide. More recent publications covering the subject of antibiotics and cancer therapy (*15–17*) have yielded no additional information that appears related to this report.

The absence of febrile reactions in 7 of the 23 tumor-bearing animals argues against a foreign protein-type response. The filtrates are apparently nonantigenic as observed by the induction of delayed hypersensitivity. On clinical grounds this presents a further argument for separating the present material from Shear's polysaccharide.

In many animals the febrile reactions do appear to be associated with impending rapid decrease in tumor size or necrosis of tumor substance. Those in which tumor regression has been slow and gradual have usually been free of febrile response. Lymphosarcoma cases have shown a striking tendency to regress rapidly and have been the most prone to develop

serious febrile reactions. While many tumor regressions have been accompanied by febrile episodes, regressions have not been dependent upon an elevated temperature. The fever seems to have been more an indicator of tumor breakdown than a necessary condition for breakdown to occur.

The filtrates have proved relatively nontoxic in the normal animal.[4] There has been no demonstrable effect on the bone marrow or white count. On the other hand, in the presence of a large mass of tumor tissue, particularly when disseminated, the filtrate is, potentially, an extremely toxic and dangerous substance. Great caution must be exerted in its use under these circumstances. Therapy should begin with very small doses and gradually increase, striving, if possible, to stay below the dose level that produces rapid destruction of tumor tissue.

Six tumor-bearing dogs, Cases #2, 3, 4, 9, 12, and 17, were treated without reactions sufficiently severe to necessitate interruption of therapy with maximum daily doses varying between 0.18 cc. and 0.25 cc. per pound. All 6 of these tumors were local growths: 3 benign, 2 malignant, and 1 of questionable malignancy. The remaining 14 dogs all experienced febrile reactions which necessitated interruption of therapy. Daily dosage per pound in these 14 dogs ranged between 0.25 cc. and 0.67 cc. Five of these cases had localized neoplasms: 4 malignant, and 1 benign. The other 9 dogs had widely disseminated disease, which was diagnosed as lymphosarcoma in 7.

In general, cats with tumors appeared to tolerate relatively larger doses than dogs. One cat with a small local neoplasm, Case #14, tolerated a maximum dose of 1.10 cc. per pound without reaction. The other two cats experienced a febrile reaction similar to that in the dog. In Case #15, the disease was apparently local but a large dose of 2.30 cc. per pound produced a febrile response. In Case #21, with generalized lymphosarcoma, a reaction was experienced after a dose of 1.00 cc. per pound. Reactions are thus seen to be generally associated with larger doses on a weight basis, but the clinical impression should be stressed that the animal with disseminated disease, particularly lymphosarcoma, is prone to experience a febrile reaction apart from the size of the dose.

Intravenous administration has proved necessary, using multiple daily injections. The impression has been that intermittent and irregular therapy is associated with unsatisfactory or slow tumor response, though controlled studies on this point are not available.

Finally, it is recognized that many cancer investigators have had little or no experience with spontaneous dog and cat neoplasms. Statistical data regarding the biological behavior of dog and cat tumors in highly inbred strains are lacking. In some ways these spontaneous tumors occurring in a randomized animal population more closely resemble the situation in human cancer than the transplantable rodent neoplasms so prevalent in research laboratories. As in human cancer, we must rely on

[4] Maximum daily doses ranged from 0.18 to 0.67 cc. per pound in the 20 dogs treated and from 1.0 to 2.3 cc. per pound in the 3 cats.

clinical experience with dog and cat neoplasms for our knowledge of the expected course to be pursued by a given tumor in a given case.

This experience has indicated that, with two or three notable exceptions, tumors in dogs and cats with histologic characteristics of malignancy may be expected to pursue a malignant course. Karlson and Mann (*18*) have stressed the benign behavior of the neoplasms referred to as transmissible venereal tumor of dogs, which occasionally is incorrectly called malignant by pathologists. The characteristic clinical features of this disease, with involvement of external genitalia, were not present in the animals of this series. Bloom (*19*), who first described mastocytoma, questions the malignancy of all cases of this disease. With the final exclusion of the lesion referred to as histiocytoma by Mulligan (*20*), which might be confused with a malignant tumor, those neoplasms that fulfill the usually histologic criteria of malignancy have behaved in the past as malignant tumors.

Heroic efforts to relieve a dog or cat from the effects of a malignant neoplasm are usually economically contraindicated and euthanasia is generally practiced. This deprives us of a large amount of statistics bearing on the life expectancy and possible rate of spontaneous regression in animals with these neoplasms. In spite of this handicap the accumulated experience of veterinarians, as reflected in the writings on veterinary medicine (*21–23*), indicates that cancer in the dog and cat has an impact like cancer in man.

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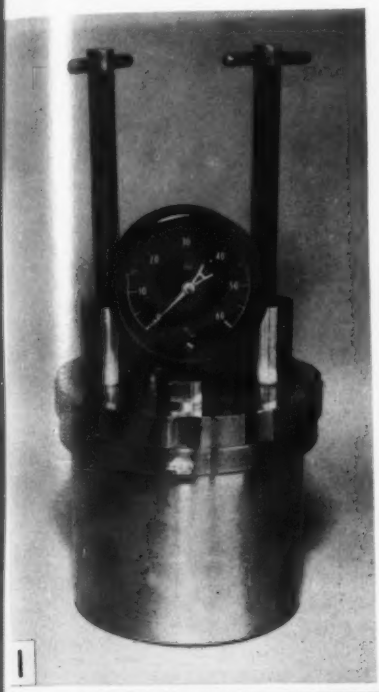
PLATE 18

FIGURE 1.—External view of fermentation chamber showing top in place with pressure gauge and pressure-regulating plungers in the withdrawn position.

FIGURE 2.—Schematic cross-section drawing of the pressure chamber showing details of construction and operation of the pressure-regulating plungers.

FIGURE 3.—Dismantled view of the pressure chamber showing the inner surface of the chamber top. The two laterally located pressure-regulating cylinders are directed toward the observer. The two fittings at the lower edge of the top were designed to accommodate *p*H electrodes. The top fitting accommodates the pressure gauge.



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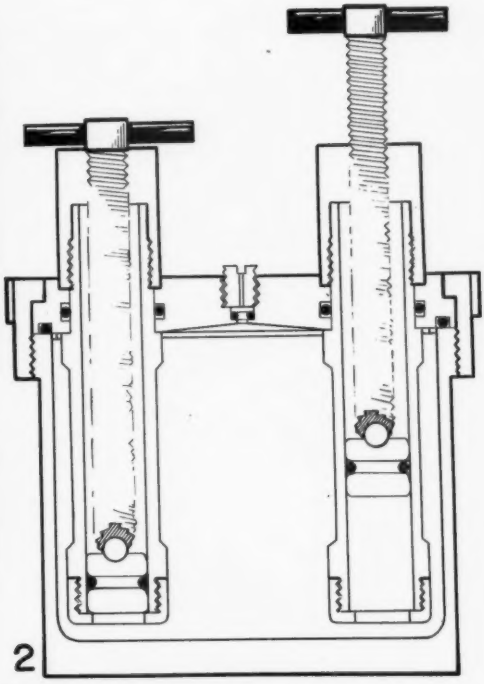

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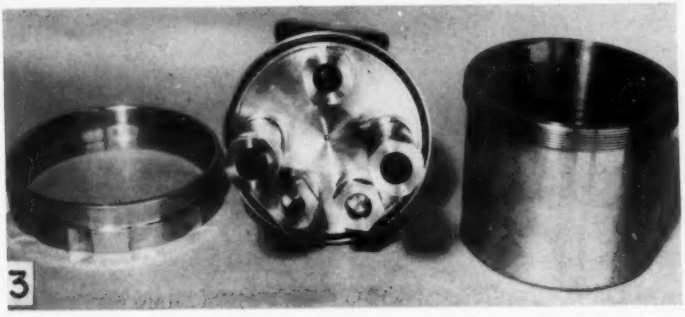

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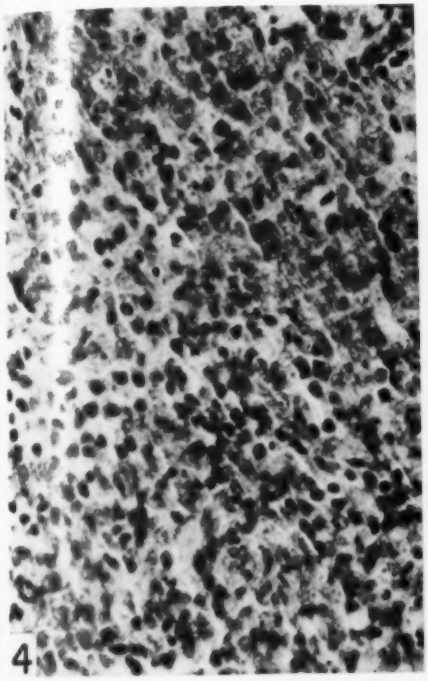
PLATE 19

FIGURE 4.—Case #1. Portions of this greatly enlarged lymph node from a mixed terrier showing generalized lymphadenopathy are replaced by overgrowth of cells with large, pale vesicular nuclei showing much mitotic activity. Hematoxylin and eosin. × 244

FIGURE 5.—Lymphoblastoma from supraclavicular lymph node at autopsy in Case #1. The node is diffusely infiltrated with cells with abundant cytoplasm and eccentrically located nuclei. Normal node architecture is extensively altered. Hematoxylin and eosin. × 122

FIGURE 6.—Kidneys that appeared grossly normal in Case #1 are focally infiltrated with immature lymphoid cells like those found in the supraclavicular lymph node (fig. 5). Hematoxylin and eosin. × 122

FIGURE 7.—Case #2. Malignant melanoma removed from the jaw of a 14-year-old black Scottish terrier. The lesion had eroded extensively the bony structure of the jaw and loosened several teeth. The pigment shown fails to stain with the Prussian-blue reaction. Hematoxylin and eosin. × 275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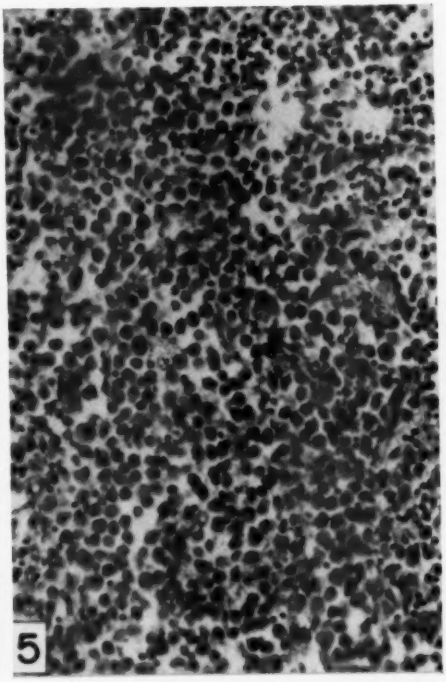
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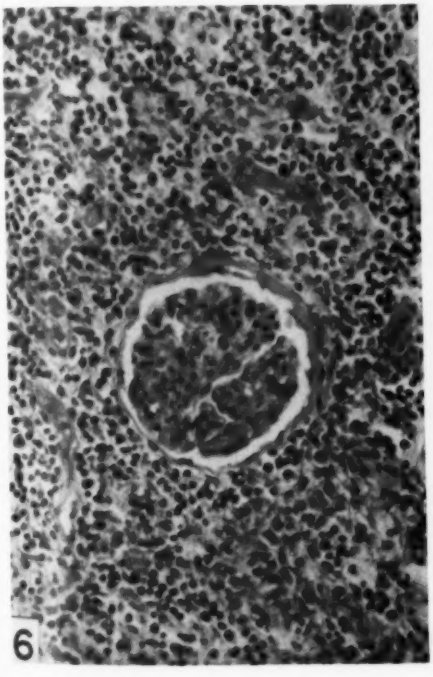
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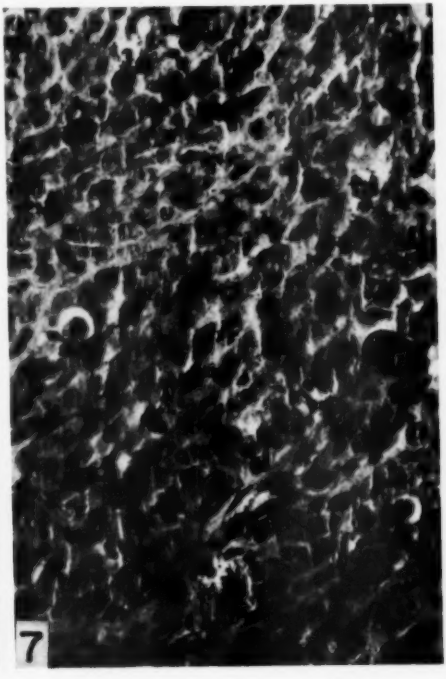
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PLATE 20

FIGURE 8.—Case #3. Infiltrating adenocarcinoma of the breast recurrent at site of surgical excision 3 weeks before. Hematoxylin and eosin. × 122

FIGURE 9.—Case #4. Adenoma of breast, or possibly low-grade carcinoma, from a cocker spaniel. Epithelial proliferation is largely intraductile or intralobular. No definite stromal infiltration is demonstrated. Hematoxylin and eosin. × 350

FIGURE 10.—Case #5. Squamous carcinoma in breast area from Dalmatian female. Hematoxylin and eosin. × 180

FIGURE 11.—Appearance of the ulcerating squamous carcinoma overlying the right inguinal breast of Case #5 midway during course of filtrate therapy. This lesion regressed approximately 50 percent in 9 months.

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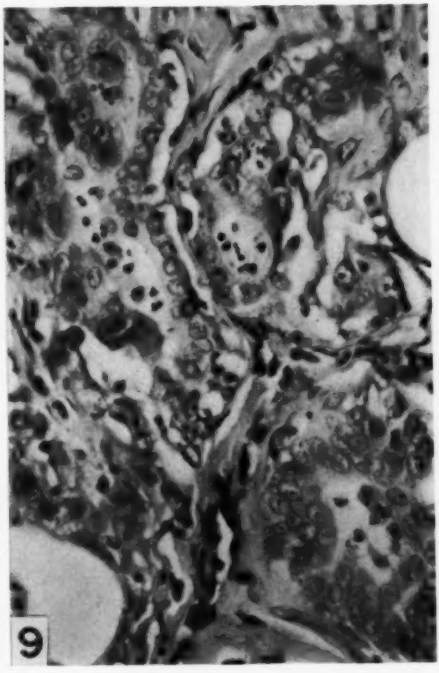
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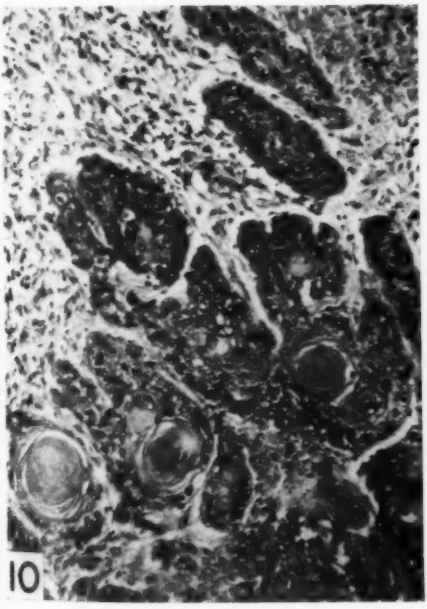
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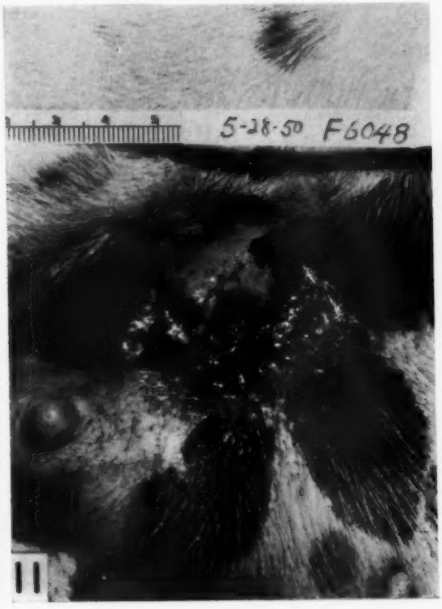
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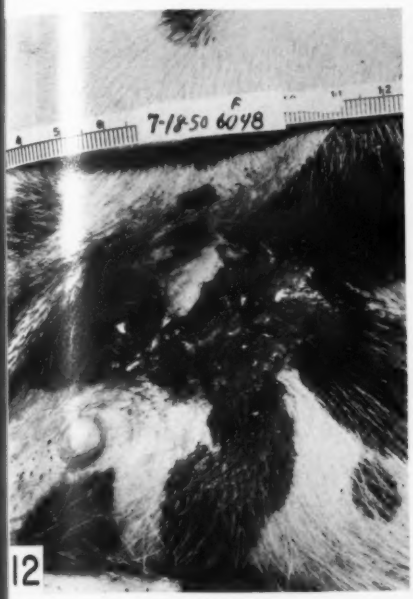
PLATE 21

FIGURE 12.—Further decrease in size of ulcerating squamous carcinoma in breast region of Case #5. This tumor rapidly increased in size when poor veins forced discontinuation of filtrate therapy.

FIGURE 13.—Case #6. Palpillary adenocarcinoma removed by biopsy from rectum of 10-year-old collie. This neoplasm extended for a distance of 10.0 cm. above the sphincter and involved three fourths of the bowel-wall circumference. Hematoxylin and eosin. × 105

FIGURE 14.—Higher-power view of rectal adenocarcinoma in Case #6. This tumor regressed and was not found at autopsy 44 months after cessation of therapy. Hematoxylin and eosin. × 210

FIGURE 15.—Case #7. Perianal adenoma with superficial ulceration from a 14-year-old cocker spaniel.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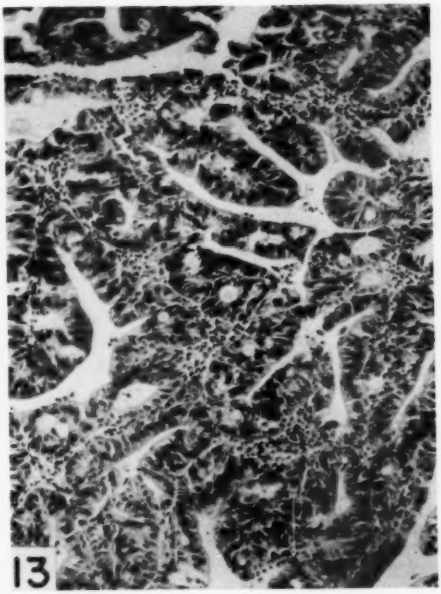
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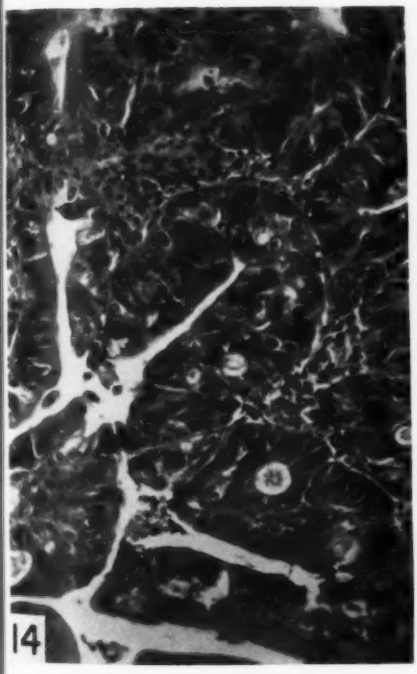
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PLATE 22

FIGURE 16.—Case #8. The characteristic involvement of the skin of the testes with mast-cell tumor infiltration. This picture was taken on September 12, 1950, before treatment was commenced.

FIGURE 17.—Elevated indurated infiltration of the skin of the thorax of the Scottish terrier in Case #8, with disseminated recurrent mast-cell tumor. This lesion is one of the 19 present before treatment was commenced.

FIGURE 18.—Superficial dermal infiltration with pleomorphic mast cells from Case #8. Hematoxylin and eosin. × 105

FIGURE 19.—Higher-power view of dermal mast-cell tumor infiltration in Case #8. The pleomorphic nature of this neoplasm possibly explains the failure of Giemsa and toluidine blue stains to reveal metachromatic granules. Hematoxylin and eosin. × 275

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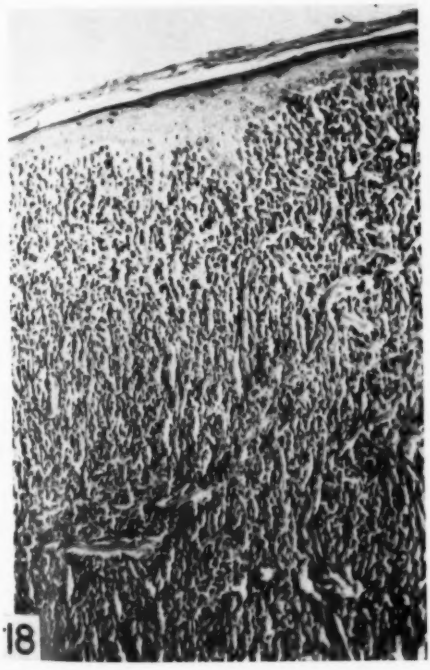

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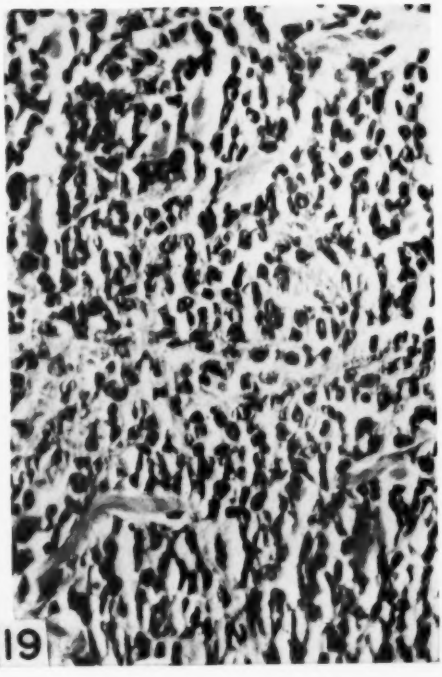

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PLATE 23

FIGURE 20.—Regression of mast-cell tumor nodule on thorax in Case #8 after 9 days of filtrate therapy. Compare with figure 17.

FIGURE 21.—Further regression of mast-cell tumor skin nodule in Case #8 shown in figures 17 and 20. The central cavitation has now healed in. Regrowth of hair is commencing.

FIGURE 22.—Decrease in the area of testicular skin involvement with mast-cell tumor after 3 weeks' filtrate therapy in Case #8. Compare with figure 16.

FIGURE 23.—Appearance of mast-cell skin nodule in Case #8 after 45 days of filtrate therapy. This is the same lesion shown in figure 16.

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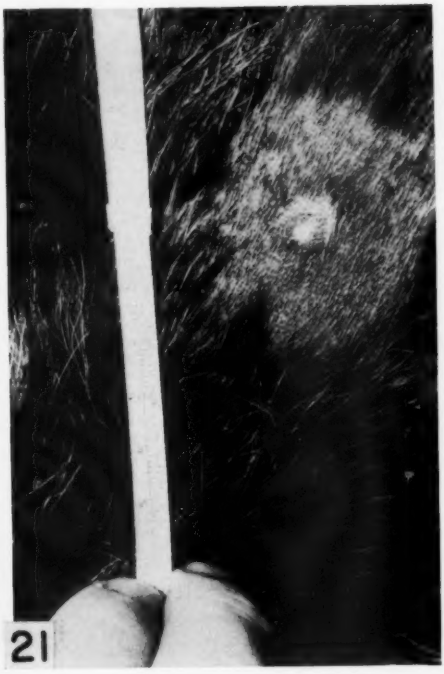
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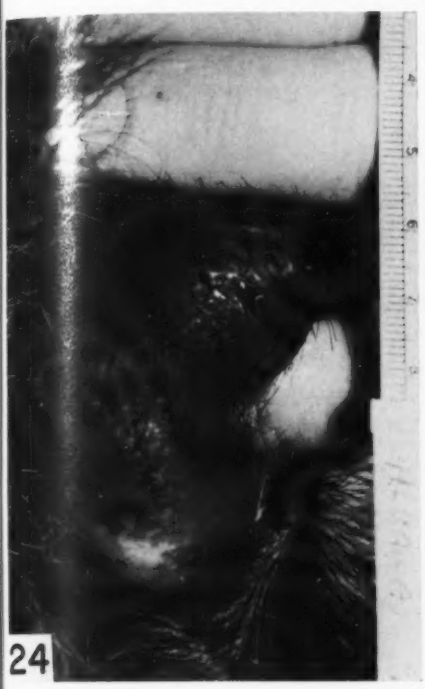
PLATE 24

FIGURE 24.—Near absence of testicular skin involvement with mast-cell tumor after 45 days of filtrate therapy in Case #8.

FIGURE 25.—Biopsy of mast-cell tumor from Case #8 at time of near complete regression. A heavy dermal infiltration with tumor cells is still evident but the cells are smaller and pyknotic. Compare with figures 18 and 19. Hematoxylin and eosin. × 210

FIGURE 26.—Appearance of testicles in Case #8 after complete clinical regression, following 60 days of filtrate therapy of mast-cell tumor. Some depigmentation of previously involved skin persists.

FIGURE 27.—Skin lesion shown in figures 17 and 20, after complete regression of mast-cell tumor in Case #8. Healing is complete but some residual depigmentation persists.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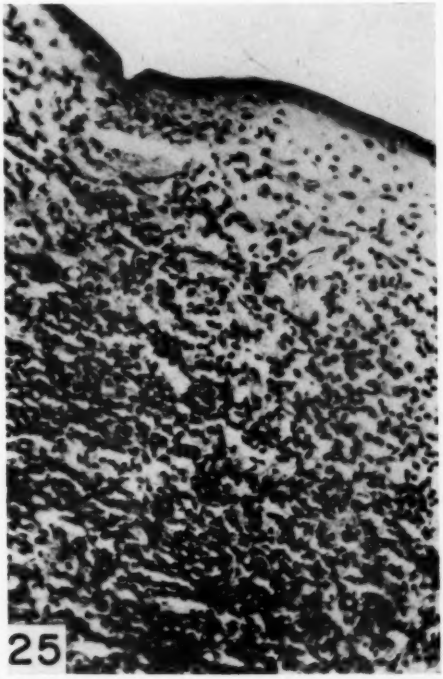
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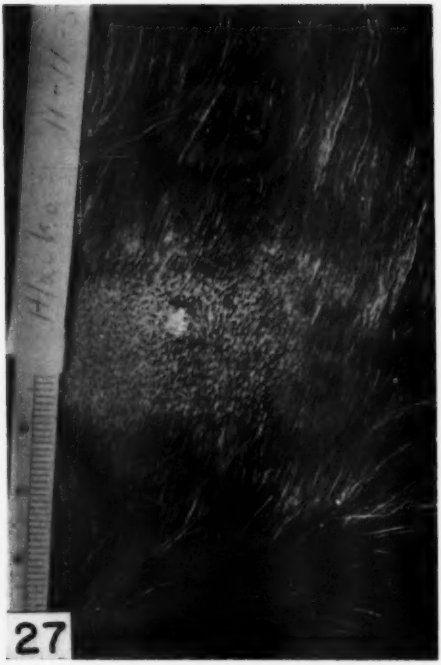
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PLATE 25

FIGURE 28.—Biopsy of site of regressed mast-cell tumor from Case #8 shown in figure 27. No tumor cells are seen. Hematoxylin and eosin. × 105

FIGURE 29.—Case #9. Perianal adenoma from cocker spaniel. The large cells with abundant granular cytoplasm and small nuclei are characteristic of this common benign neoplasm in dogs. Hematoxylin and eosin. × 350

FIGURE 30.—Perianal adenoma in Case #9 after regression to approximately one half initial size. The central area of ulceration initially occupied the whole surface of the tumor. While tumor regression progressed, the ulcerated area was drawn inward as healing proceeded about the margins.

FIGURE 31.—Case #10. Well-differentiated, actively proliferating, squamous carcinoma penetrating the deeper dermal connective tissue of the skin of the scrotum. This biopsy specimen was taken from the margin of the superficial ulcer shown in figure 32. Hematoxylin and eosin. × 210

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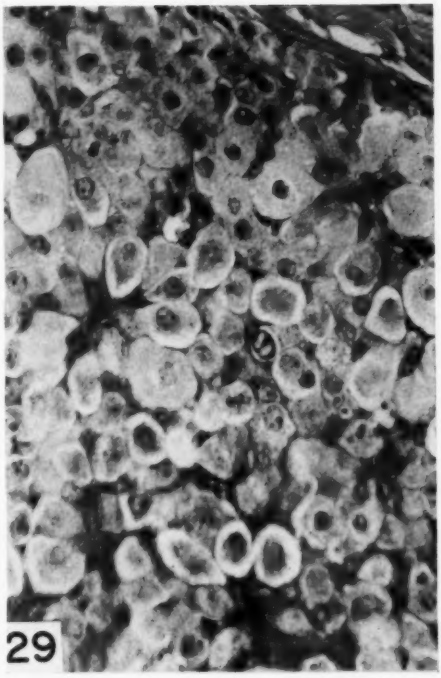
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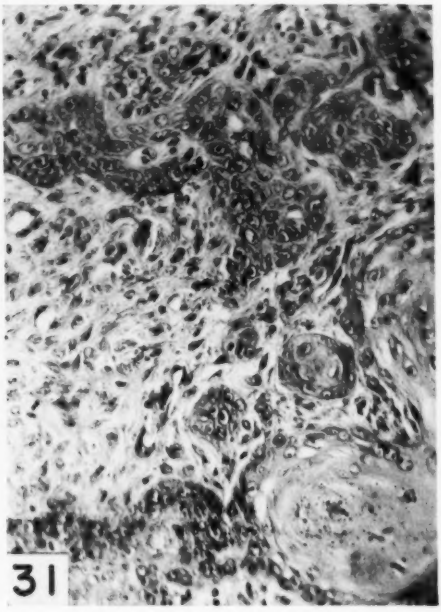
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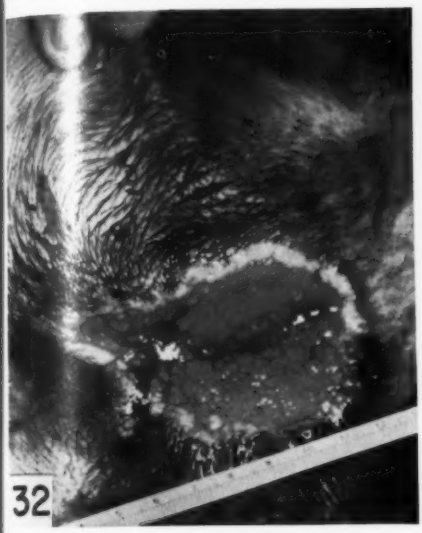
PLATE 26

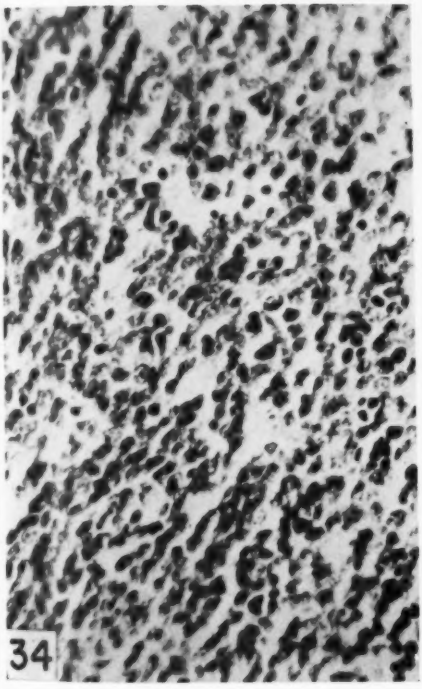
FIGURE 32.—Gaping wound following the breakdown of a surgical excision in the skin of the scrotum of Case #10. The base is composed of exuberant granulation tissue. Biopsy shown in figure 31 reveals well-differentiated squamous carcinoma.

FIGURE 33.—Partial regression of squamous-cell carcinoma of scrotum 38 days after onset of therapy in Case #10. Compare with figure 32.

FIGURE 34.—Case #11. Lymphosarcoma in a superficial, enlarged lymph node from a Scotty with generalized lymphadenopathy. Architecture of this node is entirely obliterated by infiltration with small lymphocytes. Infiltration into the surrounding soft tissue is also shown. Hematoxylin and eosin. × 244

FIGURE 35.—Case #12. Mixed tumor of the breast from a Scottish terrier. Acinarlike nests of epithelium and tissue resembling cartilage are both visible. This lesion grossly measured 8.0 × 4.0 cm. before treatment. Hematoxylin and eosin. × 350





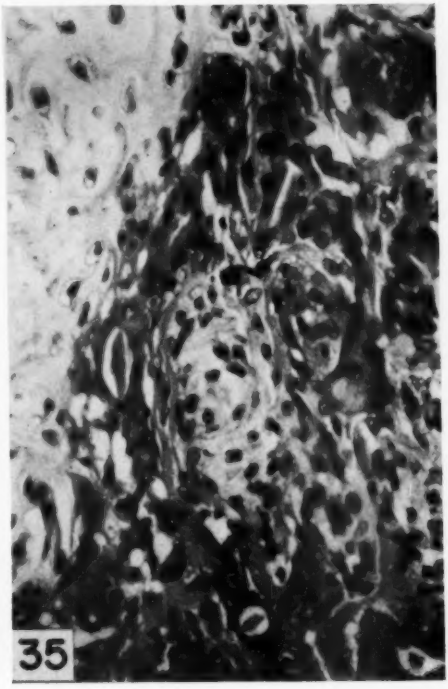


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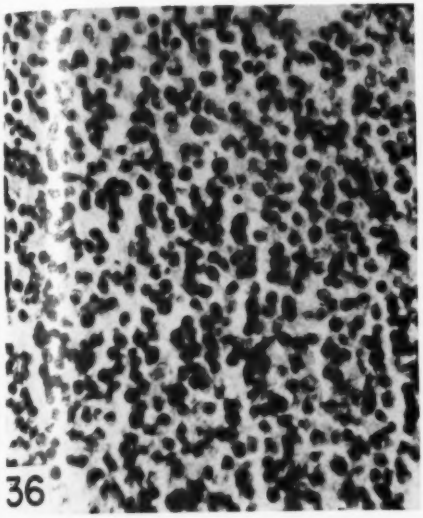
PLATE 27

FIGURE 36.—Case #13. Architecture in this superficial lymph node is entirely obliterated by proliferation of small lymphosarcoma cells. Generalized adenopathy occurred along with splenomegaly and hepatomegaly. Hematoxylin and eosin. × 244

FIGURE 37.—Case #14. Mastocytoma of right upper eyelid prior to treatment. There was no clinical evidence of disseminated disease.

FIGURE 38.—Mastocytoma of right upper eyelid of Case #14 undergoing softening after 2 weeks of filtrate therapy. The ulcerated draining area is the site of biopsy.

FIGURE 39.—Uniform, closely packed accumulation of mast cells characteristic of mastocytoma. This biopsy is from the right upper eyelid of Case #14 shown in figures 37 and 38. Hematoxylin and eosin. × 275



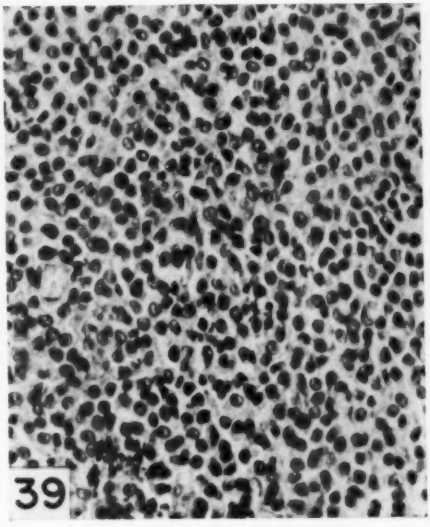


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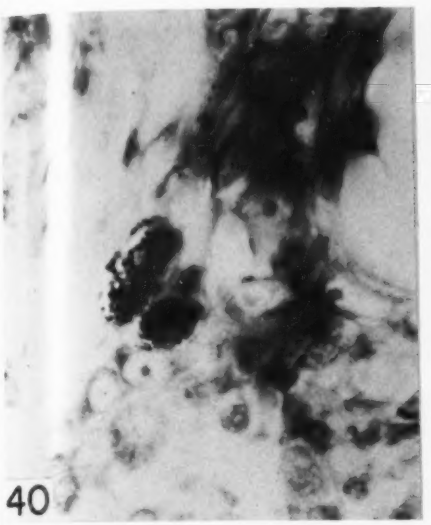
PLATE 28

FIGURE 40.—Oil-immersion view of mastocytoma of eyelid from Case #14 shown in figures 37, 38, and 39. Metachromatic mast-cell granules are clearly evident. Giemsa stain. × 1320

FIGURE 41.—Partial regression of mast-cell eyelid tumor from Case #14 after 67 days of filtrate therapy.

FIGURE 42.—Appearance of right eyelid, the site of a mast-cell tumor after 67 days of filtrate therapy in Case #14. Some scarring is seen and epilation is still evident, though the tumefaction has entirely disappeared. The cat was free of recurrence 18 months later when last seen.

FIGURE 43.—Case #15. Enlarged 6.0 cm. anterior cervical lymph node in a Siamese cat. The node was sufficiently prominent to allow it to be grasped between the fingers of the observer. There was no evidence of generalized disease of the lymph-node tissues.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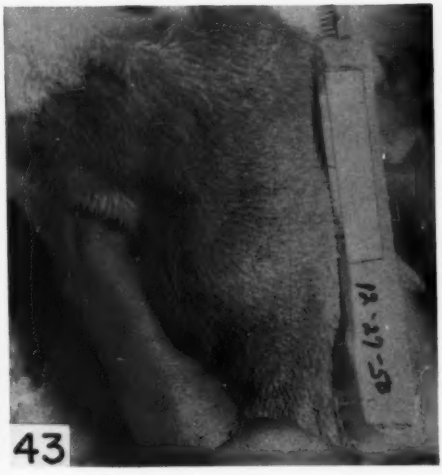
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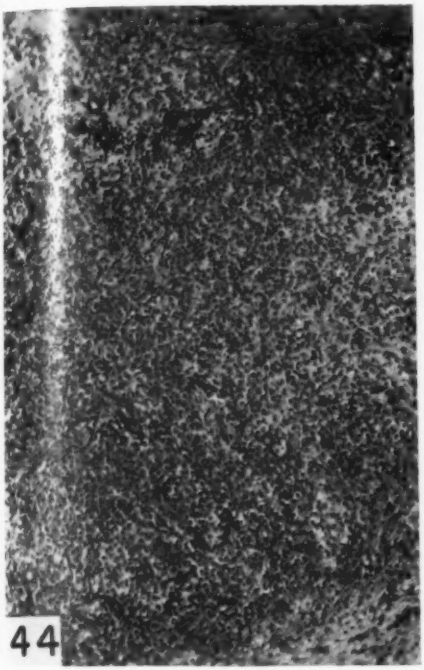
PLATE 29

FIGURE 44.—Low-power view of enlarged cervical lymph node from Case #15 shown in figure 43. Node architecture is largely destroyed by proliferation of large cells with pale, vesicular nuclei and prominent nucleoli. Hematoxylin and eosin. × 105

FIGURE 45.—Cytologic detail of obliterated portion of enlarged lymph node shown in figure 44 from Case #15. There is an active proliferation of large, pale cells of the reticulum-cell type. Hematoxylin and eosin. × 275

FIGURE 46.—Case #16. Disseminated dermal lymphosarcoma in a 7-year-old boxer showing multiple nodules of skin infiltration. Only the larger skin nodules stand out in the photograph. These lesions had appeared rapidly over a 3-week period.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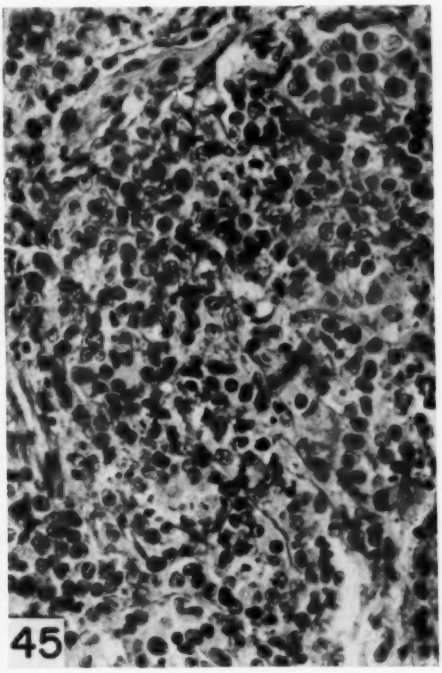
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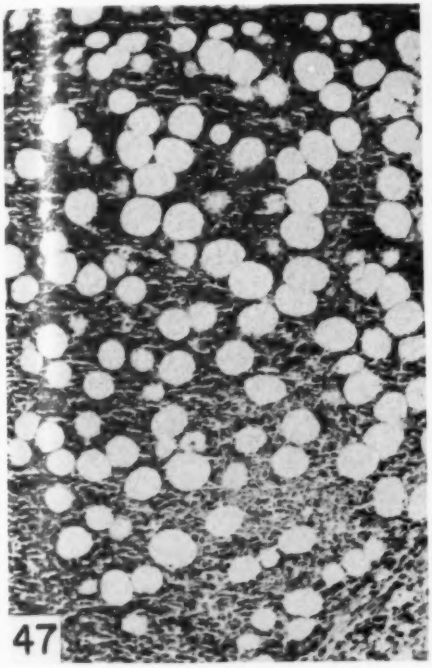
PLATE 30

FIGURE 47.—Biopsy of a skin nodule from Case #16 shown in figure 46. Mature lymphocytes are extensively infiltrating dermal adipose tissue. Surviving fat cells appear as large vacuoles in a bed of neoplastic lymphoid cells. Hematoxylin and eosin. × 105

FIGURE 48.—Partial regression of intradermal lymphosarcoma skin nodule in Case #16 during a 15-day period of filtrate therapy. The decline in prominence of nodules during this period is evident when this figure is compared with figure 46 taken before therapy.

FIGURE 49.—Areas of pigmentation indicate sites of total regression of cutaneous foci of lymphosarcoma, 43 days after onset of therapy in Case #16. The extent of the previous dermal infiltrations is accentuated by this pigmentation in a white-skin animal.

FIGURE 50.—Scattered lymphocytes remain at former site of lymphosarcoma skin nodule from Case #16 after complete clinical regression. The inflammatory or neoplastic nature of these cells cannot be ascertained with certainty for untimely death by pneumonia prevented follow-up after cessation of treatment. Hematoxylin and eosin. × 105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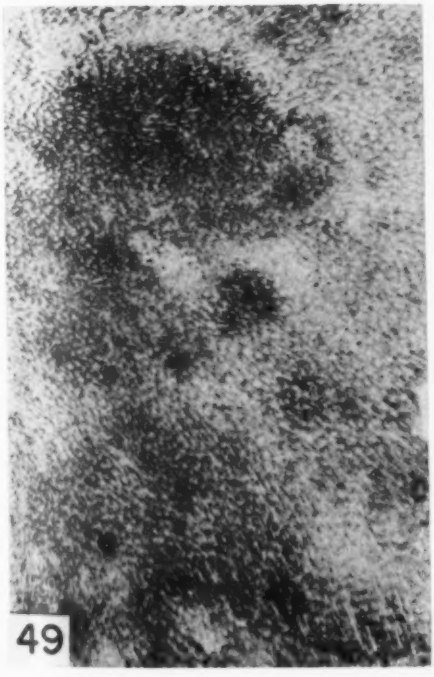
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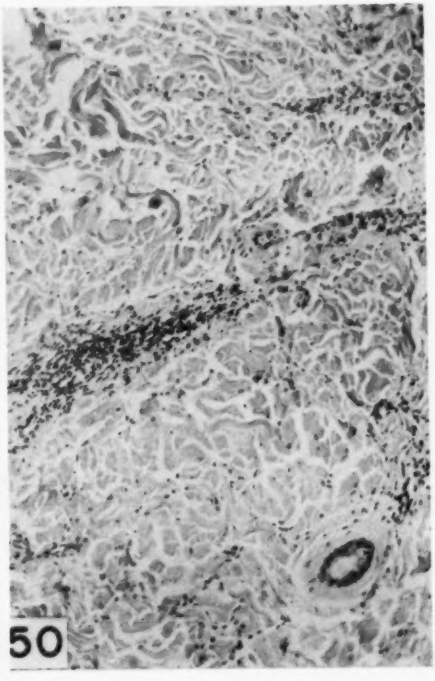
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PLATE 31

FIGURE 51.—Case #17. In 14 days tumor recurred beneath the skin and along the incision of previous surgery, and in 6 weeks reached the extent shown. The extent of the recurrence is poorly shown in this two-dimensional view. The elevated indurated region of tumor measured approximately 13.0 × 10.0 cm. and occupied most of the photographic field.

FIGURE 52.—Appearance of milk line in Case #17 after 72 days of therapy with filtrate. The large mass shown in figure 51 entirely disappeared after 20 days of therapy. Only a 2.5 cm. hard nodule alongside one nipple remains. This nodule remained stationary after 52 days of additional treatment.

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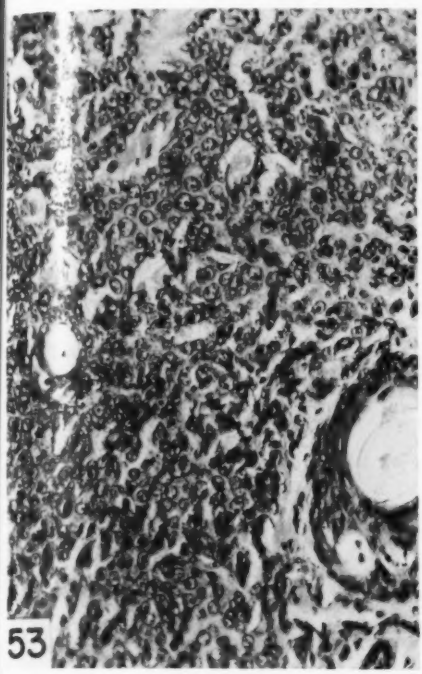
PLATE 32

FIGURE 53.—Biopsy of recurrent tumor along milk line of the cocker spaniel in Case #17 shows an undifferentiated, highly malignant neoplasm, type undetermined, possibly amelanotic melanoma. Tumor infiltration about a hair follicle is shown. Hematoxylin and eosin. × 105

FIGURE 54.—Mixed tumor of breast found at autopsy of dog, in Case #17, that died of nephritis 30 months after treatment for the malignant tumor shown in figures 51 and 53. Cartilaginous stroma and nests of epithelial cells are shown. This section is from the residual hard nodule shown in figure 52. Hematoxylin and eosin. × 180

FIGURE 55.—Skin at site of undifferentiated malignant tumor in Case #17. No tumor cells are observed, though large aggregates of melanin pigment are deposited within the superficial dermis. This pigment has suggested the possibility that the undifferentiated neoplasm was a melanoma. Hematoxylin and eosin. × 122

FIGURE 56.—Case #18. Lymphosarcoma from tissue at autopsy. Small lymphocytes are seen extensively invading the perinodal adipose tissue. Hematoxylin and eosin. × 180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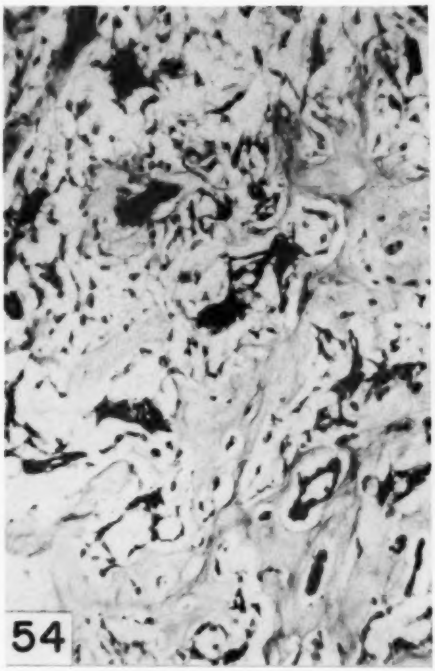
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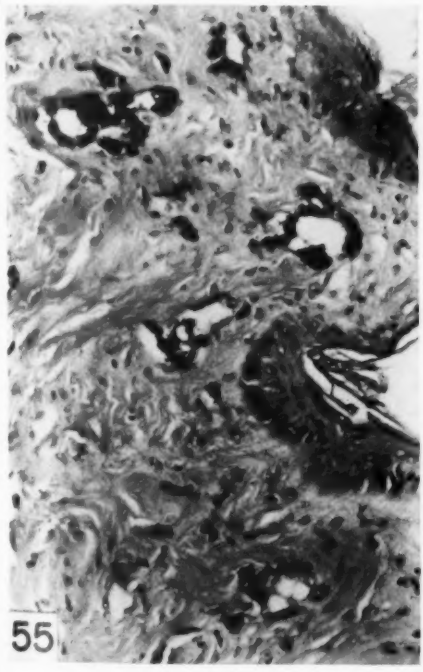
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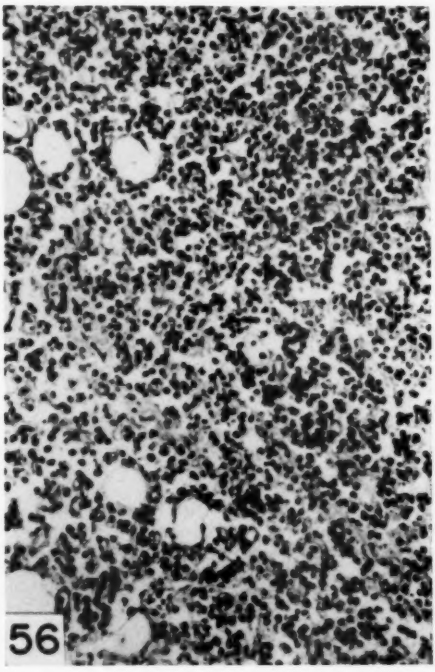
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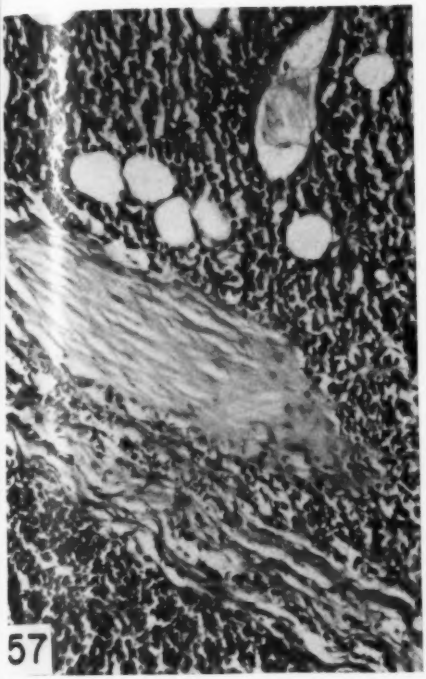
PLATE 33

FIGURE 57.—Case #19. Lymphosarcoma in a biopsy of a superficial lymph node. Lymph-node capsule transects the view diagonally. Extension of the process into the perinodal soft tissue is shown in the upper portion of the photograph. Hematoxylin and eosin. × 180

FIGURE 58.—Case #20. Lymphosarcoma in a popliteal lymph node was removed by biopsy. Infiltration of the lymph-node capsule by neoplastic cells demonstrates the malignant character of the process. Hematoxylin and eosin. × 180

FIGURE 59.—Case #21. Shaved anterior neck of cat showing protruding prominent enlargements of cervical lymph nodes. Lymph-node enlargement was generalized.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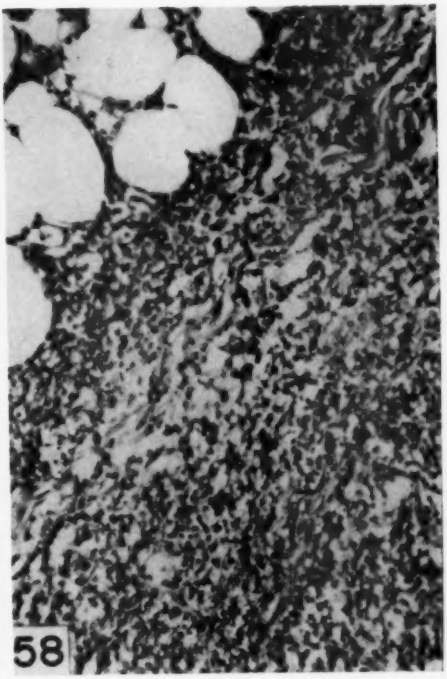
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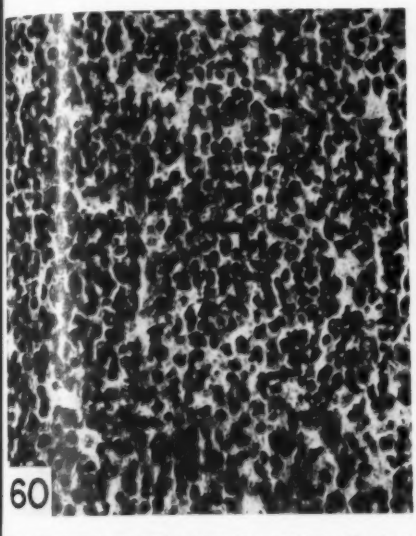
PLATE 34

FIGURE 60.—High-power view of lymph-node biopsy from Case #21 showing diffuse dense proliferation of small lymphocytes. Hematoxylin and eosin. × 275

FIGURE 61.—Normal appearance of shaved neck of cat in Case #21 after regression of enlarged lymph nodes shown in figure 59. The photograph was taken after 98 days of therapy with filtrate.

FIGURE 62.—Scarring of the liver observed in Case #21 at autopsy 41 months after regression of hepatomegaly and enlarged lymph nodes of cat shown in figures 59 and 61. Lymph nodes appeared normal at autopsy. Hematoxylin and eosin. × 122

FIGURE 63.—Case #22. Melanin pigment forming pleomorphic neoplasm from the hard and soft palate of a cocker spaniel. This tumor had metastasized to regional lymph nodes. Hematoxylin and eosin. × 180



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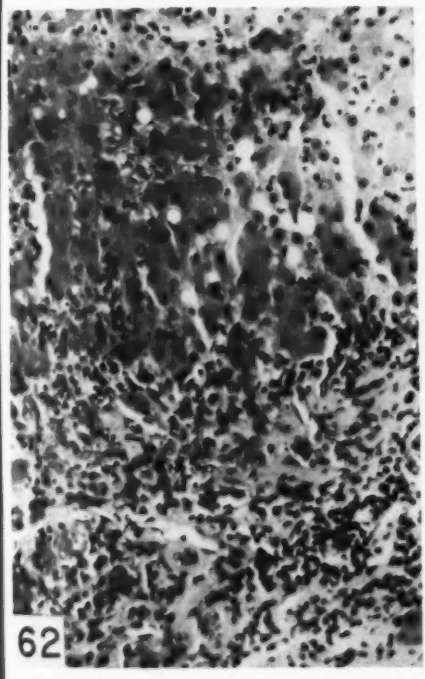

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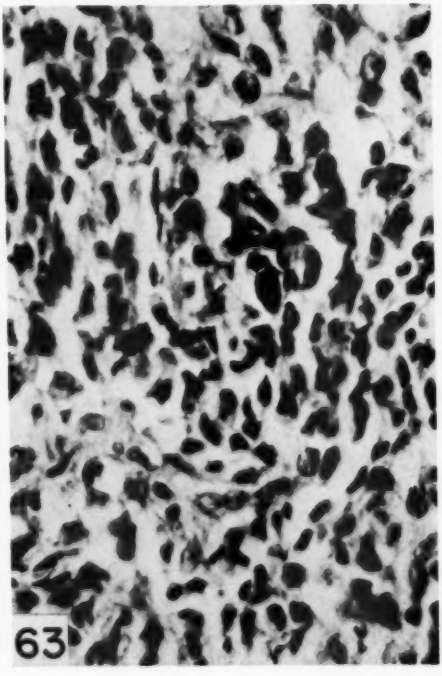

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PLATE 35

FIGURE 64.—Case #23. Elevated, soft, dark-red hemorrhagic-appearing nodule protruding from the skin of the abdomen of a mongrel dog. This lesion was the largest of several that involved the skin of this animal.

FIGURE 65.—Angiomatous nature of skin lesion in Case #23 shown in figure 64. Hematoxylin and eosin. × 105

FIGURE 66.—Higher-power view of blood vessel forming neoplasm from Case #23 shown in figure 65. The prominent and bizarre endothelial cells of the branching vascular spaces are shown. Hematoxylin and eosin. × 275

FIGURE 67.—Biopsy of hemorrhagic angiomatous skin nodule of dog in Case #23 after 16 days of filtrate therapy. The extensive infiltration of the lesion with acute inflammatory cells is evident. This inflammatory-cell reaction was not present in the biopsy taken prior to treatment, as shown in figures 65 and 66. Hematoxylin and eosin. × 105

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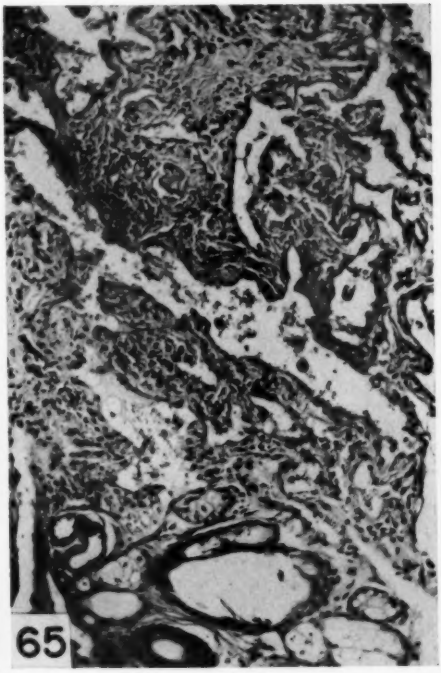

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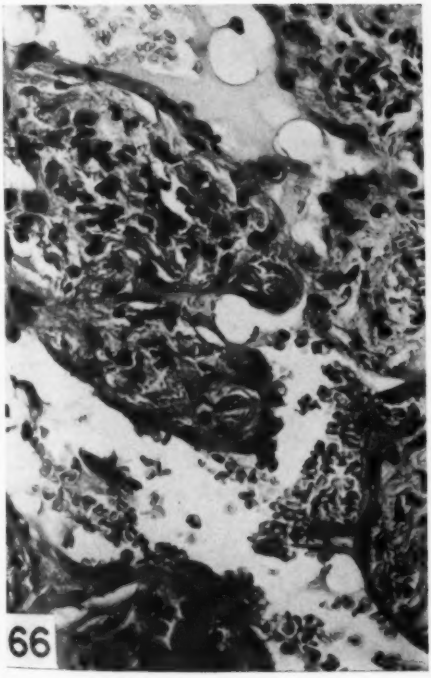

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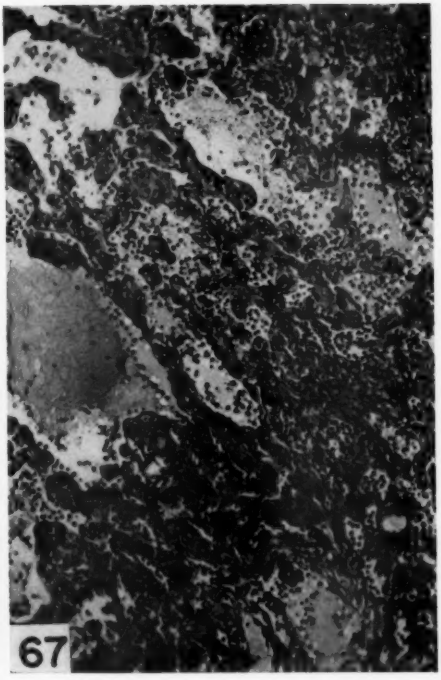

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PLATE 36

FIGURES 68, 69, and 70.—Consecutive views during an 18-day period of treatment showing progressive regression in a single skin lesion in Case #23.

FIGURE 71.—Biopsy of site of skin lesion from Case #23 shown in figure 64 after near complete regression of the lesion. Scattered lymphocytes are dispersed among small nests of endothelial cells with the appearance of young capillaries. The skin lesions in this animal entirely disappeared and did not recur for a follow-up period of 21 months. Hematoxylin and eosin. × 210

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Stray Angiosarcoma 3-4-54
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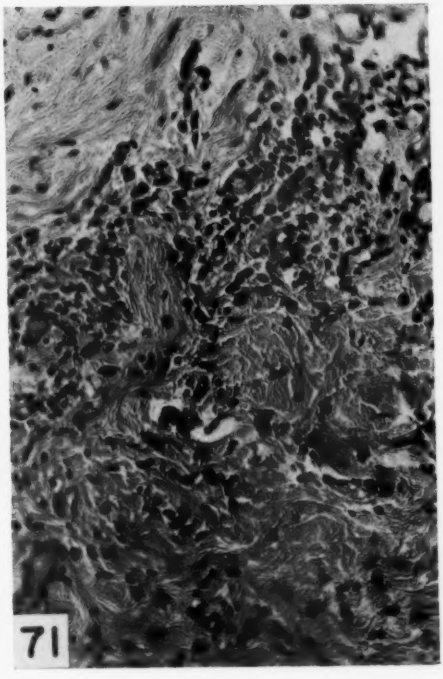
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X-Irradiation-Induced Mitotic Abnormalities in Mouse Liver Regenerating after Carbon Tetrachloride Injury. I. Total-Body Irradiation [1, 2, 3]

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Since the discovery of their profound effect upon actively dividing cells, ionizing radiations have been employed to explore the complex problems of cell growth and function. Few cytological investigations, however, have dealt with somatic animal cells. The present study concerns the effect of X rays upon the mitotic process in mature functioning cells of the mouse liver.

The liver is especially suited for such a study. Although mitotic figures are rare in adult liver, a burst of mitotic activity can be produced at any time by a variety of experimental procedures. In the present investigation CCl_4 was the stimulus employed.

In a preliminary study (*1*) it was ascertained that X irradiation had no demonstrable effect upon the extent of CCl_4 necrosis nor upon the reparation and regeneration of liver parenchyma, other than a possible slight retardation. However, the number of abnormal mitotic figures that appeared during regeneration was strikingly increased. It was emphasized that this increase in mitotic abnormalities was found after irradiation of resting cells as well as of mitotically active cells. The present paper reports the results of a quantitative study of the occurrence of these abnormal mitotic figures under the following experimental conditions: variation 1) in the dosage of X rays, 2) in the time interval between irradiation and CCl_4 injection, 3) in the time interval between irradiation and sacrifice, and 4) in the use of animals of different ages. In all experiments total-body irradiation was employed.

The liver has been described at one time or another as both a radioresistant and a radiosensitive organ. This divergence of opinion may be attributable to the difference in criteria employed in determining the response of the liver to X irradiation (*2–4*).

In the present investigation, livers of mice that had been irradiated

[1] Received for publication June 24, 1957.

[2] Presented to the Graduate School of Brown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1955.

[3] This investigation was supported in part by research grant C–510 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, administered by Professor J. Walter Wilson.

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with doses ranging from 125 to 1,000 r, but had not been treated with CCl_4, showed no morphological evidence of X-ray damage. However, morphological changes in the livers of mice were noted by Scherer (*5*) who worked within the same dose range used in this study. Warren *et al.* (*6–8*), also working within the same dose range, reported cytological changes in livers of irradiated mice. Nuclear vacuoles were observed in the liver as early as 3 hours after irradiation. These vacuoles seemed to extrude through the nuclear membranes without destroying the cell, approximately 24 hours after irradiation. Nucleoli frequently acted as foci of vacuole formation. It should be emphasized that these investigators used a freeze-drying technique for their tissue sections while ordinary fixing and staining procedures were used in the present study.

Wilson and Stowell (*9*), using higher X-ray doses (5,000 to 12,000 r) and irradiating only the liver region, were able to observe morphological changes in the livers of mice. They noted a decrease in cell size, and attributed it to loss of lipide material, while both nuclei and nucleoli increased in size. Isolated necrotic cells and areas of necrosis and hemorrhage were found, most often in the portal areas.

A temporary suppression of the mitotic rate in livers of rats irradiated at intervals before and after partial hepatectomy has been reported by Williams *et al.* (*10–12*). Colchicine was used, however, so that abnormal mitotic figures could not be studied.

Brues and Reitz (*13*) have compared the effects of total-body X irradiation, injection of Pu^{239} and P^{32}, and continuous exposure to glass beads containing carrier-free Y^{91}. All irradiation treatments were given 24 hours after partial hepatectomy. The livers were examined 48 hours later. Irradiation increased the number of abnormal mitotic figures and inhibited mitosis. In the present study, interest was centered on exploring the effects of irradiation on a resting rather than a mitotically active tissue.

Materials and Methods

1) *Animals.*—The animals used in this investigation were albino mice of the BUB strain, an inbred strain developed in this laboratory. Except when designated, only male mice, 60 to 90 days of age, were used. They were fed Purina laboratory chow. Food and water were available at all times. The mice were maintained in air-conditioned rooms kept at about 78° F.

2) *Carbon tetrachloride treatment.*—All animals treated with CCl_4 received the same dose, which was 0.1 cc. of a 40 percent solution of CCl_4 in sesame oil, administered subcutaneously. All CCl_4 injections were administered between 9 and 11 a.m.

3) *Irradiation procedure.*—The source of X rays for all experiments was a Picker-Waite therapy machine. The conditions were as follows: 200 kv., 20 ma., a filter of ½ mm. Cu and 1 mm. Al, with 240 r per minute delivered in air at a distance of 20 cm. from the tube.

All irradiation was carried out during the morning hours, if possible

between 9 and 11 a.m. (*14*). The animals were irradiated in groups of 3, in a circular styron container, 4 inches in diameter and 1 inch in depth. It was placed in a wooden phantom that was designed to distribute the backscatter evenly.

4) *Histological techniques.*—In all routine experiments, the mice were sacrificed during the morning hours. They were stunned with a sharp blow on the head, decapitated, and allowed to bleed thoroughly. The tip of the left lateral lobe of the liver was removed and fixed in Bouin's fluid for 24 hours, dehydrated through dioxane, embedded in paraffin, sectioned at 5μ, and stained in Delafield's hematoxylin and eosin.

5) *Method of analysis.*—While mitotic abnormalities were observed in all stages of mitosis, only abnormalities occurring in anaphase and telophase, *i.e.*, chromosome bridges, were chosen for quantitative study, since abnormalities in these stages were relatively easy to recognize, thus minimizing the subjective element. The difference between normal and abnormal mitotic figures in other stages is much less obvious.

The extent of X-ray injury was expressed as the percentage of all anaphases and telophases observed to be abnormal, *i.e.*, percentage of abnormal figures equals A/T where "A" denotes the number of abnormal anaphase and telophase figures, and "T" denotes the total number of anaphase and telophase figures, both normal and abnormal.

Mitotic counts were performed with an oil-immersion lens at a magnification of 970 diameters, and all figures from late prophase to middle telophase were counted and classified. After a total of 100 mitotic figures had been examined, another section on the same slide was chosen and the procedure repeated, bringing the total to 200 mitotic figures per animal. The duplicate determinations of A/T percent for each animal agreed quite well, as did counts from different animals receiving the same treatment.

Results

Control Animals

Two control groups of mice were used: one received X-ray treatment and the other CCl_4 injection. X-ray doses ranging from 125 to 1,000 r produced no observable cytological effects. On the other hand, following CCl_4 treatment, from ¼ to ½ of the central region of each lobule was necrotic and eosinophilic. Mitotic figures were scattered at random throughout the intact parenchyma. Abnormalities were rare; the average was 1 percent for 24 control animals. These occurred in metaphase, anaphase, and telophase, and were like the abnormalities observed after irradiation, but they were, in general, less severe.

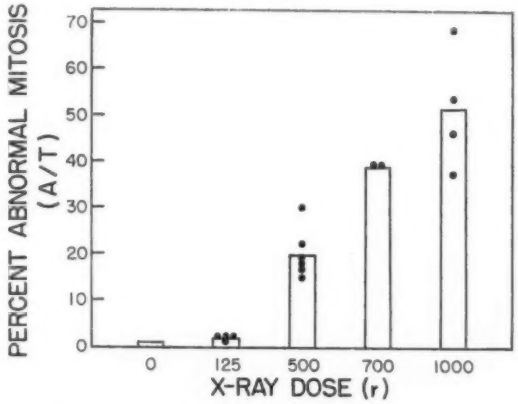
Cytological Effects of X Irradiation Combined with CCl_4 Injection

In all animals receiving both X-ray and CCl_4 treatment, there was a marked increase in the number of mitotic abnormalities compared to CCl_4-treated controls. There were many abnormal metaphases: lagging chromosomes were sometimes at the periphery of the cytoplasm remote from the spindle, or the spindle itself was malformed. In the latter,

there was a tendency for tripolar formation or a complete lack of polarity, so that chromosome orientation was haphazard. It would be impractical to describe all the diverse kinds of abnormal figures occurring in anaphase and telophase. They varied from figures with the most tenuous bridges to figures with broad bands of material between the daughter nuclei. The number of bridges ranged from 1 to 4 or more. Frequently, no clear-cut nuclear separation occurred. The two daughter plates, solidly fused together, presented the typical dumbbell-shaped appearance which is often designated as amitosis. Finally, there were mitotic figures so severely disarranged that they defied description. Micronuclei were also observed.

X Irradiation Preceding CCl_4 Treatment

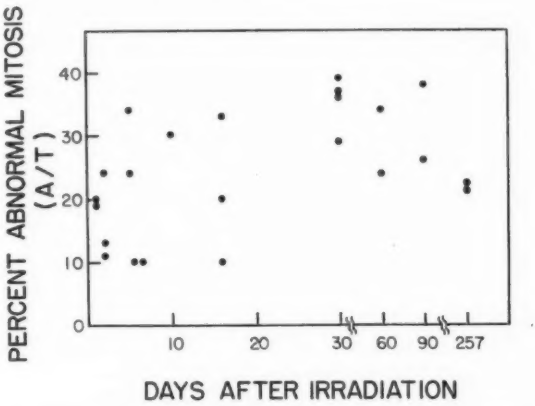
A) Irradiation immediately preceding CCl_4 injection.—CCl_4 injections were administered within 5 to 10 minutes after the animals had been irradiated with doses ranging from 125 to 1,000 r. The results are shown in text-figure 1. With increase in the X-ray dose, the percentage of abnormal mitotic figures increased: 125 r, 2 percent; 500 r, 20 percent; 700 r, 39 percent; 1,000 r, 52 percent. In general, at the higher dose levels the bridges were thicker and more numerous.



TEXT-FIGURE 1.—Effect of varying X-ray dosage upon percentage of mitotic abnormalities (number of abnormal anaphases and telophases over the total number of anaphases and telophases) in mice injected with CCl_4 following irradiation. Each point represents the results for a single animal based on a total count of 200 mitotic figures. Bar height indicates the group average; 0 r is the average for 24 animals receiving only CCl_4 injection. Animals were sacrificed 2 or 3 days after irradiation.

B) Irradiation followed by CCl_4 injection from 1 to 257 days later.—In this group of animals the time of CCl_4 injection varied from 1 to 257 days after irradiation (500 r). The results support the conclusion that once the liver has been irradiated, there is no recovery indicated in the percentage of abnormal mitotic figures obtained when mitotic activity is induced

by CCl_4. As shown in text-figure 2, the intervals between irradiation and CCl_4 injection were 1, 2, 5, 6, 10, 16, 30, 60, 90, and 257 days. The percentage of abnormal mitotic figures for each animal was 19, 20; 11, 13; 24, 34; 10, 10; 30; 10, 20, 33; 29, 36, 37, 39; 24, 34; 26, 38; 21, 22; grouped for the corresponding time interval.



TEXT-FIGURE 2.—Persistence of mitotic abnormalities following irradiation (500 r; liver resting at the time of irradiation). Graph gives the time interval between irradiation and CCl_4 injection. Animals were sacrificed 2 or 3 days later. Each point represents the results for a single animal based upon a total count of 200 mitotic figures.

X Irradiation Following CCl_4 Treatment

X Irradiation 1 or 2 Days after CCl_4 Injection

A) Animals sacrificed 1 or 2 days after irradiation.—Animals in these experiments were irradiated with 500 r either 1 day after CCl_4 injection—before the onset of mitosis—or 2 days after injection when mitotic activity was at its peak. No significant difference could be discerned between the results obtained on these 2 days (table 1). Although the number of animals in each group is small, the results suggest that animals irradiated at both times have a somewhat greater percentage of mitotic abnormalities (33 and 29%, respectively) than animals irradiated when their livers are mitotically inactive (20%), as shown by the group of animals receiving 500 r in text-figure 1.

B) Animals sacrificed 32, 33, 62, or 92 days after irradiation.—These animals received the same treatment as those in section *A* except the interval after irradiation was extended. It was necessary therefore to inject a second dose of CCl_4 to produce mitotic figures for counting. The animals were sacrificed 2 or 3 days later. As in the case of animals irradiated when their livers were mitotically inactive (text-fig. 2), the percentage of mitotic abnormalities did not diminish with time. The time between irradiation and sacrifice was 1, 2, 32, 33, 62, and 92 days,

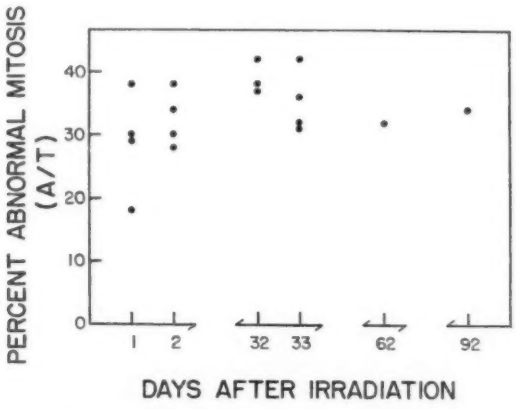
TABLE 1.—*Effect of time variation between CCl_4 injection and irradiation upon the percentage of mitotic abnormalities*

Time between CCl_4 injection and irradiation (*days*)	Time between irradiation and sacrifice (*days*)	Abnormal anaphases and telophases (*percent*)	Total anaphases and telophases (*percent*) *	Abnormal anaphases and telophases ($A/T\%$) †
1	2	16	57	28
1	2	18	60	30
1	2	23	69	34
1	2	28	74	38
				Avg. 33
2	1	12	63	18
2	1	17	58	29
2	1	16	53	30
2	1	24	63	38
				Avg. 29

* The total count of mitotic figures was 200 per animal.

† All percentages except A/T percent were referred to total number of mitotic figures; A/T percent $= 100 \frac{\text{No. of abnormal anaphases and telophases}}{\text{Total no. of anaphases and telophases}}$.

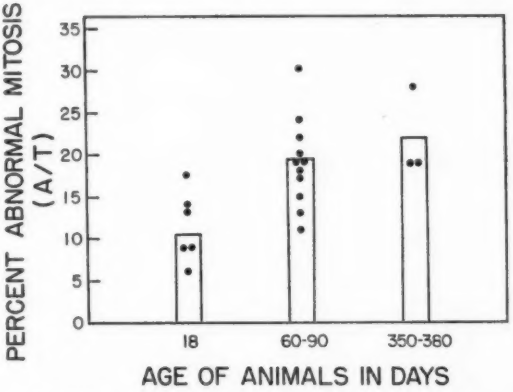
and the percentage of abnormal mitotic figures for each animal was 18, 29, 30, 38; 28, 30, 34, 38; 37, 38, 42; 31, 32, 36, 42; 32; 34; grouped for the corresponding time intervals (*see* text-fig. 3).



TEXT-FIGURE 3.—Persistence of mitotic abnormalities following irradiation (500 r; liver active at the time of irradiation). Each animal was given a CCl_4 injection, irradiated 1 or 2 days later, and sacrificed the specified number of days after irradiation. Except for the 1- and 2-day groups, a second CCl_4 injection was administered 2 or 3 days before sacrifice. Each point represents the results for a single animal, based upon a total count of 200 mitotic figures.

Irradiation of 18-Day-Old, Young Adult, and Year-Old Animals

Young mice 18 days of age and young adults were irradiated with 500 r and injected with CCl_4, either immediately or 1 or 2 days following irradiation. (The data on young adult animals include 6 animals shown in text-fig. 1.) Since, as shown in text-figure 2, there is no change with time after injection, these animals could be compared to a small group of animals, approximately 1 year old, which were immediately injected with CCl_4. The 18-day-old animals are less affected by 500 r (11%) than either young adults (19%) or "year-old" animals (22%) (text-fig. 4).



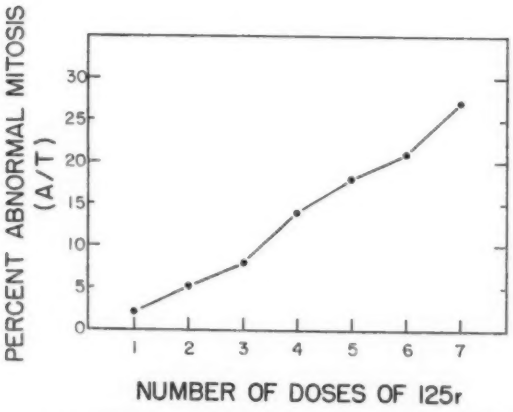
TEXT-FIGURE 4.—Correlation of age of animal with percentage of mitotic abnormalities. Each animal was injected with CCl_4 shortly after irradiation (for the 18-day-old animals 0, 1, or 2 days after irradiation) and sacrificed 2 or 3 days after injection. Each point represents results for a single animal, based upon a total count of 200 mitotic figures.

Repeated Doses of Irradiation

In the "long-term" experiments described, the effect of X rays was long-lasting. This observation suggested the possibility of a cumulative effect.

A) Successive doses of 125 r at daily intervals.—Each animal was irradiated daily for 4 days. After the last dose of 125 r, the mice were immediately injected with CCl_4 and sacrificed 2 or 3 days later. The X-ray effects were found to be cumulative: 4 animals receiving 1 dose of 125 r averaged 2 percent mitotic abnormalities (text-fig. 1) and 6 animals receiving 4 daily doses averaged 9 percent.

B) Successive doses of 125 r at 5- to 7-day intervals.—In this group of animals the time interval between X-ray doses was extended from 5 to 7 days. CCl_4 was injected immediately after the last X-ray dose and the animals were sacrificed 2 or 3 days later. The results indicate that as the number of doses of 125 r increases, the percentage of mitotic abnormalities increases (the number of doses of 125 r ranged from 1 to 7; the corresponding percentages of abnormal mitotic figures were 2, 5, 8, 14, 18, 21, and 27%; *see* text-fig. 5).



TEXT-FIGURE 5.—Cumulative effect of repeated doses of 125 r administered at 5- to 7-day intervals upon percentage of mitotic abnormalities. CCl_4 injection followed the last irradiation and sacrifice was 2 or 3 days later. Each point, except for the 1-dose case, represents an average for 2 animals; the 1-dose point, an average for 4 animals. A total count of 200 mitotic figures was made for each animal.

Discussion

The effects of ionizing radiation on chromosomes have generally been divided into two classes: 1) those designated as "primary," "physiological," or "nonlocalized," which lead to alteration in the surface properties of the chromosomes resulting in stickiness and clumping, and 2) those ionizing effects designated as "secondary," "structural," or "localized," which are thought to be direct in that actual breakage of the chromosome is produced by an ionizing particle passing through or close to it at the point where the breakage occurs. It is frequently thought that the "structural" effects occur in cells that enter division after the period of mitotic depression, while the "physiological" effects are exhibited by cells in actual mitosis at the time of irradiation. "Physiological" effects are considered temporary; "structural" changes, permanent (*15*).

Unfortunately the abnormal mitotic figures, *i.e.*, bridges, resulting from either "physiological" or "structural" effects were indistinguishable in the present study. The occurrence of micronuclei was of no aid in distinguishing between the two types of bridges, because micronuclei may arise from isolated chromosomes that have failed to be incorporated in the spindle as well as from acentric fragments in chromosome breakage. Probably both "physiological" and "dicentric" bridges occur, but it is impossible to estimate in what proportion and to determine whether there is any alteration in the proportion under different experimental conditions.

Prevailing opinions (*16–18*) concerning the time factor for "physiological" bridge formation would seem to rule out the occurrence of this type of bridge in the present investigation. Presumably the conditions leading to "physiological" bridges are transient, persisting for only a short time after irradiation and only in cells that are in division at the time of X irradia-

tion. In the present study the animals were sacrificed 2 and 3 days after irradiation at a time when such physiological effects would be absent; and in the majority of experiments the cells were mitotically inactive at the time of irradiation.

Whatever the origin of these bridges, a consideration of the following evidence suggests that the X-ray injury measured in this study is comparable to the injury resulting from changes in chromosome structure: 1) X-ray damage was dose dependent. 2) X-ray injury was independent of dose fractionation. 3) There appeared to be no recovery from X-ray injury during the time intervals studied.

The time of irradiation relative to CCl_4 injection appeared to be a factor in determining the extent of irradiation damage. Although the number of animals is small, livers irradiated while mitotically inactive exhibited fewer mitotic abnormalities (20%) than livers irradiated 1 day after CCl_4 injection (33%), before the onset of mitosis, or 2 days after CCl_4 injection, at the height of mitotic activity (29%). These results are complicated by the fact that the mitotically inactive liver is undamaged at the time of irradiation, whereas livers irradiated 1 and 2 days after CCl_4 injection contain a large number of necrotic cells. Breakdown products from necrotic cells in the central areas or direct injury to the remaining cells in the portal areas may enhance X-ray effects. The picture may be further complicated by the occurrence of a large number of cells in preprophase 1 day after CCl_4 treatment.

Abrams (*19*) has studied the influence of age and body weight on the susceptibility of mice to the lethal effects of X irradiation (550 r), and found no significant differences in survival. In the present study, 18-day-old mice had fewer mitotic abnormalities than adults. Possibly the mitotic observations are more sensitive. Some possible reasons for this difference in response of the different age groups are that in regard to the liver itself, the liver-cell nuclei are much smaller and more numerous in juvenile mice than in adults. There is also a difference in chromosome number, young animals having preponderantly diploid nuclei, while adults have a fairly large percentage of polyploid nuclei (*20*). Bishop (*21*) presents evidence that chromosome breakage is proportional to the chromosome number, which could account for the greater number of bridges in adult liver.

Two points seem worthy of special emphasis: 1) radiation injury was inflicted in the majority of cases at a time when the liver was mitotically inactive, and 2) this injury persists over a prolonged period. Two possible explanations as to how this injury could persist are that either the individual cells persist bearing the original X-ray injury, or the damage is transmitted unchanged in frequency through subsequent cell divisions.

The question of the importance of nuclear integrity for cell maintenance and function can now be raised. A large percentage of the liver cells after X irradiation must not have a completely balanced complement of functioning chromosomes, yet they appear capable of carrying on their numerous functions indefinitely. Only when the liver cells are

forced to divide is X-ray injury apparent (*22*). Errera (*23*) has cautioned, "This does not necessarily mean that the DNA-containing constituents are more sensitive to ionizing radiations than others, but effects on DNA constituents may perhaps be more conspicuous because of their controlling role on cellular processes and perhaps their greater specificity." The results of this investigation appear to bear out this statement.

Summary

1) The effects of X rays upon mitosis initiated in the liver of the mouse by CCl_4 injection have been studied. Total-body irradiation (125–1,000 r) produced no visible cytological changes in the liver. In all livers stimulated to mitosis, however, X rays had a profound effect upon mitotic figures, regardless of whether the livers were irradiated before or after the initiation of mitosis. Despite the large number of abnormal mitotic figures produced, CCl_4 damage was not enhanced and liver repair was not pronouncedly affected by X irradiation.

2) The mitotic abnormalities studied consisted of bridges of chromatin material between the separating plates. The bridges varied in length, width, and number of strands present.

3) The extent of X-ray injury to mitosis was expressed in percentage of A/T; where "A" denotes the number of abnormal anaphase and telophase figures, and "T" the total number of anaphase and telophase figures.

4) As the X-ray dose was increased, there was a concurrent increase in the percentage of abnormalities: 125 r, 2 percent; 500 r, 20 percent; 700 r, 39 percent; 1,000 r, 52 percent.

5) The intensity of the effect was not diminished with passage of time: The percentage of mitotic abnormalities obtained when cells were induced to divide by administration of CCl_4, 257 days after irradiation, agreed closely with the figures obtained when animals were similarly treated and sacrificed 2 days after irradiation.

6) The time of irradiation with respect to CCl_4 injection determined to some degree the percentage of mitotic abnormalities produced. Irradiation 2 or 3 days after CCl_4 injection resulted in a slightly higher percentage of mitotic abnormalities than when CCl_4 injection followed irradiation.

7) Eighteen-day-old animals averaged 11 percent abnormalities compared to 20 percent for a similarly treated group of adult animals.

8) The effect of repeated small doses (125 r) was cumulative, whether the animals were irradiated daily or at 5- to 7-day intervals.

9) The conclusions reached were: 1) Resting liver is only slightly less sensitive to X irradiation than regenerating liver; 2) X-ray effects are prolonged and persist, but are brought to light only if the cells are forced to divide.

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X-Irradiation-Induced Mitotic Abnormalities in Mouse Liver Regenerating after Carbon Tetrachloride Injury. II. Partial-Body Irradiation [1,2,3]

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In a previous study of the effects of total-body irradiation on the liver of the mouse, the X-ray damage was evaluated by recording the percentage of abnormal mitotic figures produced among regenerating liver cells following injury resulting from CCl_4 injection (*1*). A question that naturally arises from such an investigation is how much of the X-ray effect measured was actually due to direct irradiation of the liver itself and what part, if any, was due to the systemic effect of irradiation of other parts of the mouse. The results of the present study in which various types of shielding were employed indicate that the systemic factor is important in determining the extent of X-ray injury.

Materials and Methods

1) *Animals.*—Male albino mice of the BUB strain, 60 to 90 days of age, maintained as decribed previously (*1*), were used throughout these experiments.

2) *Carbon tetrachloride treatment.*—All animals received subcutaneously 0.1 cc. of a 40 percent solution of CCl_4 in sesame oil as described previously (*1*). Animals were sacrificed 2 or 3 days after injection.

3) *Nembutal® treatment.*—In all experiments involving partial-body irradiation, animals were anesthetized with Nembutal (0.98 mg. of sodium pentobarbital/gm. body weight) except that the dose was reduced by approximately one half for those animals treated 1 or 2 days previously with CCl_4, because they were more susceptible to the action of this drug.

A brief investigation indicated that Nembutal had no effect upon CCl_4 necrosis or subsequent mitosis and an insignificant effect upon the percentage of mitotic abnormalities occurring after irradiation.

[1] Received for publication June 24, 1957.

[2] Presented to the Graduate School of Brown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1955.

[3] This investigation was supported in part by research grant C-510 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, administered by Dr. J. Walter Wilson.

[4] The author is indebted to Dr. J. Walter Wilson for constructive criticism and constant encouragement, and to Dr. Nancy L. R. Bucher for invaluable aid during the final preparation and revision of the manuscript.

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4) Irradiation procedure.—The procedure was the same as previously described (*1*), except that the dose rate was 290 r per minute delivered in air at a distance of 17 cm. from the tube. The animals were irradiated individually under Nembutal anesthesia during the morning hours. Appropriate regions were shielded with lead plates 2 mm. thick, held in place with scotch tape.

In shielding experiments involving the exteriorized left lateral lobe, the lobe was delivered through a small abdominal incision in the left upper quadrant. The liver lobe was never touched; by simply pressing on the abdominal wall bilaterally, about three quarters of the left lateral lobe was extruded. Once out, it did not slip back unless too large an incision had been made. With the animal on its side, a lead shield was placed over the entire animal except for the exposed lobe; or, in reverse-shielding experiments, the lead shield was placed over the exteriorized lobe—leaving the rest of the animal exposed to irradiation. After X-ray treatment, the incision was quickly sutured and the animal kept warm under an electric bulb until the effects of the Nembutal had worn off.

5) Histological techniques and method of analysis.—These procedures have been described previously (*1*).

Results

Shielding Experiments Involving Intact Mice

A) Liver region exposed to irradiation.—In this group of animals, the head and tail regions were shielded and the liver region irradiated with 700, 1,000, or 1,500 r. CCl_4 was injected immediately after irradiation. The mice were sacrificed 2 or 3 days later. The results are shown in table 1. The percentage of abnormal mitotic figures increased as the X-ray dose increased: 700 r, 30 percent; 1,000 r, 42 percent; 1,500 r, 67 percent.

B) Liver region shielded from irradiation.—The shielding was reversed in this group of experiments; that is, the liver region was shielded while the head and tail areas were irradiated. X-ray treatment was given under two sets of conditions: 1) when livers were mitotically inactive (CCl_4 injected immediately after irradiation) and 2) at the height of mitosis (2 days after CCl_4 injection). The X-ray dose in each case was 1,000 r. The percentage of mitotic abnormalities under either set of conditions was small compared to values obtained with this dose in total-body irradiated animals [(*1*), *see* table 1].

Shielding Experiments Involving Exteriorized Left Lateral Lobe

The liver region also includes parts of many other organs. The secondary effects that irradiation of these contiguous regions might have had upon the liver could not be adequately evaluated. Experiments were therefore undertaken in which the liver alone was treated.

A) Irradiation of tip of the left lateral lobe in mitotically inactive liver.—The distal third of the exteriorized left lateral lobe was irradiated with 500 r, while the rest of the animal was shielded. The animals were in-

TABLE 1.—*Comparison of the percentage of abnormalities in exposed and shielded liver regions of X-irradiated mice*

X-ray dose (*r*)	Time between CCl_4 injection and irradiation* (*days*)	Abnormal anaphases and telophases (*percent*)	Total anaphases and telophases (*percent*)†	Abnormal anaphases and telophases (*A/T* %)‡
Liver region irradiated; head and tail regions shielded				
700	0	18	63	30
700	0	17	59	29
1000	0	30	74	41
1000	0	31	74	42
1500	0	37	57	65
1500	0	50	73	68
Liver region shielded; head and tail regions irradiated				
1000	0	1	71	1
1000	0	1	79	1
1000	0	2	66	2
1000	0	1	51	2
1000	0	1	66	2
1000	0	2	52	4
1000	0	5	78	6
1000	0	4	65	6
1000	2	3	79	4
1000	2	5	66	8
1000	2	10	80	12
1000	2	8	68	12

*Animals sacrificed 2 or 3 days after CCl_4 injection.
†The total count of mitotic figures was 200 per animal.
‡All percentages except A/T percent were referred to total number of mitotic figures;

$$\text{A/T percent} = 100\,\frac{\text{No. of abnormal anaphases and telophases}}{\text{Total no. of anaphases and telophases}}.$$

jected the following day with CCl_4 and sacrificed 2 or 3 days later. They were in excellent condition at the time of CCl_4 injection and showed no ill effects from the operation.

The results appear in table 2. A low percentage of mitotic abnormalities was found in the irradiated areas. The values found in the shielded areas of the same lobes were within the range for nonirradiated CCl_4-treated control rats (*1*). Cytological observations of the remaining lobes of the liver agreed well with those from the shielded proximal portions of the left lateral lobe. Only results of mitotic counts from the left lateral lobe are included in the present study.

B) Irradiation of tip of left lateral lobe in the mitotically active liver.—In this group of animals, CCl_4 was injected 2 days before the operation and X-ray treatment (700 r). Otherwise, the procedure followed was the same as in the preceding group. The results were the same; the percentages of mitotic abnormalities were slightly higher in the irradiated tip than in the shielded proximal portion of the left lateral lobe (table 3).

C) Tip of left lateral lobe shielded.—In this group, CCl_4 injection preceded irradiation by 2 days and the shielding was reversed; that is, all of the

TABLE 2.—*Comparison of the percentage of abnormalities in shielded anterior section and in irradiated tip of the mitotically inactive left lateral lobe (CCl_4 injected 1 day after irradiation with 500 r)**

Region of left lateral lobe of liver	Abnormal anaphases and telophases (*percent*)	Total anaphases and telophases (*percent*)†	Abnormal anaphases and telophases (*A/T%*)‡
Irradiated	2	65	3
Shielded	1	55	2
Irradiated	2	79	3
Shielded	1	78	1
Irradiated	3	77	4
Shielded	1	76	1
Irradiated	8	60	13
Shielded	1	71	1

*Animals sacrificed 2 or 3 days after CCl_4 injection.
†The total count of mitotic figures was 200 per animal.
‡All percentages except A/T percent were referred to total number of mitotic figures;

$$\text{A/T percent} = 100\ \frac{\text{No. of abnormal anaphases and telophases}}{\text{Total no. of anaphases and telophases}}.$$

TABLE 3.—*Comparison of the percentage of abnormalities in shielded section and in irradiated tip of the mitotically active left lateral lobe (CCl_4 injected 2 days before irradiation with 700 r)*

Region of left lateral lobe of liver	Time between irradiation and sacrifice (*hours*)	Abnormal anaphases and telophases (*percent*)	Total anaphases and telophases (*percent*)*	Abnormal anaphases and telophases (*A/T%*)†
Irradiated	1¼	4	51	8
Shielded	1¼	2	56	4
Irradiated	6½	4	66	6
Shielded	6½	1	65	2
Irradiated	24	2	52	4
Shielded	24	0	54	0
Irradiated	24	5	56	9
Shielded	24	1	61	1
Irradiated	24	4	63	6
Shielded	24	1	53	2

*The total count of mitotic figures was 200 per animal.
†All percentages except A/T percent were referred to total number of mitotic figures;

$$\text{A/T percent} = 100\ \frac{\text{No. of abnormal anaphases and telophases}}{\text{Total no. of anaphases and telophases}}.$$

animal except the tip of the left lateral lobe was irradiated with 700 r. The results are presented in table 4. All the irradiated sections of the liver contained a high percentage of mitotic abnormalities. The shielded tip was relatively free from abnormalities.

TABLE 4.—*Comparison of the percentage of abnormalities in irradiated section and in shielded tip of the mitotically active left lateral lobe (CCl_4 injected 2 days before irradiation with 700 r)*

Region of left lateral lobe of liver	Time between irradiation and sacrifice (*hours*)	Abnormal anaphases and telophases (*percent*)	Total anaphases and telophases (*percent*)*	Abnormal anaphases and telophases (*A/T%*)†
Irradiated	1	15	90	17
Shielded	1	1	77	1
Irradiated	24	16	63	25
Shielded	24	1	64	2
Irradiated	48	19	50	38
Shielded	48	3	57	5

*The total count of mitotic figures was 200 per animal.
†All percentages except A/T percent were referred to total number of mitotic figures;

$$\text{A/T percent} = 100\,\frac{\text{No. of abnormal anaphases and telophases}}{\text{Total no. of anaphases and telophases}}.$$

Discussion

No morphological differences were observed between the abnormal mitotic figures resulting from total-body X irradiation and those occurring in animals whose livers were shielded at the time of irradiation. Similar types of abnormal figures are found in mouse livers that have not been subjected to irradiation. They have been observed in livers in which mitosis has been induced by a variety of means, including CCl_4 injection without irradiation, and also in the livers of young mice in which mitosis has not yet ceased (*2*). The percentage of abnormalities in these non-irradiated animals is usually small, but in rare instances it has been surprisingly large. It is an open question whether the pathways by which these abnormalities are generated are in part, at least, like the course by which X rays produce mitotic abnormalities.

In this paper we have attempted to evaluate the relative importance of local and systemic irradiation effects in producing these mitotic abnormalities. Other investigators have observed changes in the liver after irradiation of other areas of the body. Ross and Ely (*3*) irradiated rats with X-ray doses of 1,000 r and found that the amount of hepatic glycogen increased in fasting animals. This increase in hepatic glycogen could not be accounted for by direct irradiation alone, since an increase occurred even when the liver region was protected with lead shields. Scherer (*4*), in an extensive study of irradiation effects on the liver of the mouse, used leg and head irradiation and observed, in the liver cells of each animal, morphological changes in the chromatin network, shape and number of nucleoli, nuclear size and density, and number of binucleate cells. The magnitude of change was the same as with total-body irradiation, which is contrary to the findings of the present investigation.

Shaver (*5*), in work on the testes of the rat, has made observations comparable to those in this study. He found that total-body irradiation with

500 r caused definite cytological changes in the testes, while the same dose, also total-body with testes and inferior extremities shielded, or local irradiation of the testes, brought about no significant change in the testes. These results must be interpreted with caution for just the opposite results have been reported by Kohn (*6*) who, with smaller X-ray doses (60 r to 210 r), observed that the dose to the testis determined the changes occurring in the organ, while irradiation of the remainder of the body had little, if any, effect on the testis.

In experiments in which the liver region was irradiated, while the head and tail were shielded, the percentage of mitotic abnormalities, though lower, paralleled similarly treated animals that received total-body X irradiation. In the reverse experiments, in which the liver region was shielded, the percentage of mitotic abnormalities was markedly lower, suggesting that the greater percentage of abnormal mitotic figures was produced by direct irradiation of the liver. When the liver region is irradiated, other important body organs are also irradiated, particularly the spleen, which is known to be important in irradiation injury and recovery. In these intact animals, therefore, the systemic reaction could not be evaluated, but the intensity of effect was less than in animals receiving total-body irradiation.

In order to reduce the systemic effect to a minimum, since it can never be entirely eliminated, shielding experiments were carried out on the exteriorized left lateral lobe of the liver. When only the tip of this lobe was irradiated, the percentage of mitotic abnormalities observed in the exposed area was low. This may at first seem surprising in view of the results from experiments in which direct irradiation of the entire liver region produced a large percentage of mitotic abnormalities.

In regard to this point, the results of Gershbein's investigation (*7*) would appear to substantiate the finding that direct irradiation of the liver produces relatively little effect. In one instance he irradiated the remaining liver lobe of rats, after partial hepatectomy, with X-ray doses up to 20,000 r (the remainder of the animal shielded) and found that liver regeneration did not differ statistically from that in control rats. In a second study, Gershbein and Krotoszynski (*8*) found that irradiation of the entire liver of rats with 6,500 r produced no marked alteration in the succinic dehydrogenase and nucleic acid levels. They point out that these results are in direct contrast to those of Di Bella (*9*), who found marked decreases in succinic dehydrogenase levels 24 or 48 hours after irradiation (600 and 800 r). Di Bella, however, employed *total-body X irradiation*, which in the light of the results of the present investigation may well account for the discrepancy.

Anoxia should be mentioned as a factor that may reduce the percentage of mitotic abnormalities observed in experiments involving exteriorization of the liver, because anoxia is known to afford some protection from irradiation. The fact, however, that in the experiment in which the tip was shielded and the remainder of the animal irradiated the percentage of mitotic abnormalities was comparable to that found in intact animals,

in which the liver region was shielded, would seem to rule out the possibility of anoxia as a protective factor here.

Possibly the results can be explained as follows: in animals receiving total-body X irradiation, the entire body is injured. Toxic substances may be built up in the liver cells themselves. In addition, there is an influx into the liver of breakdown products from the remainder of the body. Thus, in measuring the injury to the liver in animals exposed to total-body irradiation, one obtains the combined effect of irradiation on the entire liver and the systemic effect from the remainder of the body (*1*). In experiments in which the head and tail regions are shielded, the irradiation effect measured includes that of direct irradiation of the liver and a reduced systemic effect. In the case of irradiation of the left lateral tip, the systemic effect can probably be regarded as negligible, so that only the effect of X rays on liver tissue alone is being measured.

In conclusion, direct irradiation of the liver alone does not result in a large percentage of mitotic abnormalities nor does an intense systemic effect lead to many abnormalities; however, when direct irradiation is combined with irradiation of other areas of the body, a synergistic response occurs that leads to a large percentage of mitotic abnormalities.

Summary

1) A series of studies was carried out with various kinds of shielding to determine whether mitotic abnormalities observed in the liver were due to direct action of X rays, secondary systemic effects, or a combination of both.

(a) In experiments in which the liver region was irradiated while the head and tail regions were shielded, the percentage of mitotic abnormalities, though somewhat lower, paralleled results for animals exposed to total-body X irradiation.

(b) In the reverse experiments, in which the liver region was shielded, the percentage of mitotic abnormalities was very low.

(c) In more precise shielding experiments, animals were operated upon and the left lateral lobe of the liver exposed and irradiated; or the left lateral lobe was shielded while the remainder of the animal was irradiated. In both instances, few abnormalities were observed in the tip of the exteriorized liver lobe.

2) Results of the above experiments indicate that although direct irradiation is necessary for obtaining a high percentage of abnormalities, little damage occurs unless other areas of the animal have been irradiated. This constitutes strong evidence for the importance of a humoral factor or factors in irradiation injury.

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The Appearance and Disappearance of Antibodies to Rous Virus in Normal Chickens [1,2]

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The filterable nature of the chicken sarcoma has been recognized for almost 50 years (*1*), but its natural history is still obscure. There are several reasons for this and one is that neutralization methods have only recently been able to handle the large numbers of chicken sera required for testing antibody response to naturally acquired infection. Further, most of these tests including a recent modification of Duran-Reynals' test (*2*) are directed against the capacity of the virus to produce tumors. This reduces the accuracy unless large numbers of chicks or balanced inocula are used (*3*) and may subject the test itself to false interpretation because of antibody against the tumor cell instead of virus (*4*). Sarcoma virus is hard to recover from some tumors even when it is experimentally inoculated (*5*). Finally, no continued studies on experimental flocks have been undertaken.

It is known that asymptomatic and apparently healthy chickens may have neutralizing substances that prevent the development of tumors when a mixture of virus and serum are inoculated (*6*). This presumptive antibody is produced in response to the injection of virus (*7*); it may persist for as long as 2 years (*8*), and like other antibodies is transmissible passively through the yolk to the chick (*9*).

This study of the appearance, prevalence, and disappearance of antibody against the Rous virus was encouraged by the fact that we had been able to measure virus activity (*10*) by its capacity to produce the hemorrhagic disease first described by Duran-Reynals (*11*). The hemorrhagic activity could be neutralized by sera from chickens immunized against the virus (*10*). This then should measure only the neutralization of the virus and not antibody to tumor cells. The neutralizing activity when quantitated had the usual attributes of antibody.

The present study reports the prevalence of antibodies in a flock of chickens about 6 months old, and the absence of antibodies in these chickens by the 12th to 24th month. Antibody was determined both by random sampling within the flock and by testing individual chickens over a

[1] Received for publication July 19, 1957.

[2] Supported by research grant C-1230 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

2-year period. A series of baby chicks exposed to the "infected" flock acquired antibodies, while a small series raised in isolation did not.

Method

The original White Leghorn flock, 8 hens and 1 rooster about 6 months old, was acquired from the same local poulterer whose embryonated egg stock was used for the titration of the virus and its antibody. The flock was put indoors in a small room where nests were made and where the flock remained for the duration of the study. Each chicken was banded, and the eggs from individual chickens were marked just after they were laid. Chicks from individual hens were individually recorded, one half being kept in the room with the original flock, the other half in a separate building which had no other chickens.

Sera were obtained either by cardiac puncture or by bleeding from a wing vein. A standard dilution of 10 ID50's of virus was used for the neutralization tests, which were done with tenfold dilutions of sera previously inactivated at 56° C. for 30 minutes. The standard dose of virus was obtained by using 1 vial of a pool of previously titered infected chick-embryo liver, which was kept frozen in dry ice or in an electric deepfreeze at −40° C. The sera and virus were incubated for 1 to 2 hours at 4° C., and then inoculated intravenously into 10-day-old chick embryos. Control embryos were inoculated with a combination of virus and a one-tenth dilution of heated sera of human placental cord, which in repeated tests has failed to show any neutralizing activity. The embryos were incubated at 39° to 40° C. and observed daily. Embryos found dead within the 1st day were discarded as traumatic losses, and the rest were examined for hemorrhagic lesions. The 50 percent dilution activity of the serum was determined by calculating the amount of serum which theoretically was necessary to reduce the percentage of hemorrhagic lesions from 85 to 100 percent to 50 percent. Such calculations were of course justifiable only if a clear-cut end point of neutralization had been obtained. A sample neutralization result and the calculation from it is given in table 1. A neutralization of 0 to 1 logarithmic units was considered as negative, 1.0 to 1.4 as doubtful, and of 1.5 or more as positive.

Since a series of inocula control material showed that 10 ID50's actually produced 85 percent infection instead of the usual 100 percent, all figures

TABLE 1.—*Neutralization of 10 chick-embryo ID50 doses of Rous virus by chicken sera*

Serum dilution	Chicken		Control (*human cord serum*)
	#82	#59	
10^{-1}	6/7*	1/7	3/5
10^{-2}	6/6	0/4	
10^{-3}	7/7	1/6	
10^{-4}	6/7	4/7	
10^{-5}	2/4	5/6	

*Six of the 7 inoculated embryos showed hemorrhagic lesions.

were corrected for this and accumulated according to the Reed Muench method. The accumulation was done first and the percentage of embryos *with* lesions was calculated. This percentage was multiplied by 1.2 to bring 85 to 100 percent (102 percent). The 50 percent end point was determined from these corrected figures. For chicken #59 it was $10^{3.8}$; the uncorrected figure would have been $10^{3.6}$. The neutralization test with serum #82 was negative.

Results

There is recent evidence that neutralizing substances which inhibit the production of tumors may have a large component of antibodies to cells (*4*). There is also a historic dispute concerning the relation of virus to the normal constituents of a cell (*12*). We will therefore review the evidence of the specificity of our neutralization test: (*a*) In this test we determine the neutralization activity against a destructive effect of the virus, presumably not against new growths (*10*). (*b*) The characteristics of the neutralizing substance are those of antibody, *i.e.*, heat stability and dilution-neutralization ratios (*10*). (*c*) Of 15 chickens that lacked antibody when inoculated with Rous virus, 4 developed antibody 1 month later, 4 at 2 months, and 7 failed to develop antibody in 2 months. (*See* text-fig. 1 for summary of data. Although the relationship of each chick to its dam is indicated in text-fig. 1, the individual chicks were not kept with their own mother exclusively when they were kept with the flock. The isolated group were put in a room in another building, but, again, they were kept together in a small flock.) (*d*) In table 2 we have compared the results of our neutralization tests on sera from the Regional Poultry Research Laboratory, Agricultural Research Service, Animal and Poultry Husbandry Research Branch, U. S. Department of Agriculture, East Lansing, Michigan, with data furnished by Dr. B. R. Burmester of that laboratory. The only neutralization in our series was in the susceptible line 6, which developed antibodies in their experiments as well (*13*).

TABLE 2.—*Neutralizing antibody (hemorrhagic lesions) in random sera from different lines of chickens at East Lansing Agricultural Experimental Station*

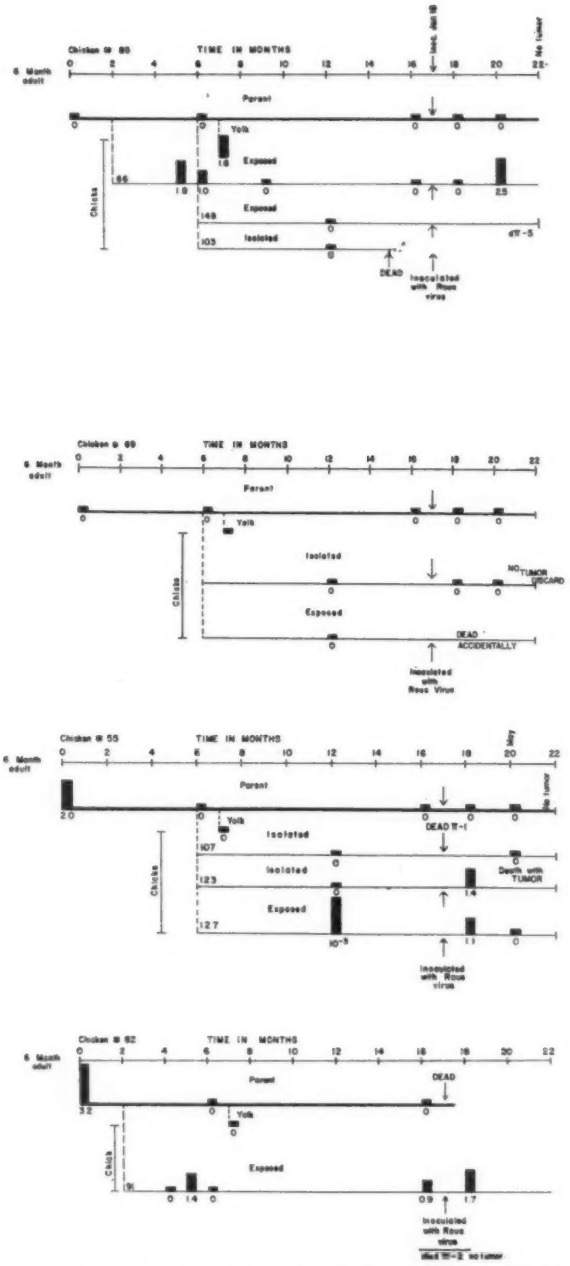
Line	Susceptibility *	Neutralization†	Neutralization of hemorrhagic lesions
6	78. 4	51/57	3/6
7	0. 0	1/29	0/6
15	44. 3	—	
15I	88. 7	—	0/11

*Courtesy Dr. B. R. Burmester, of the U. S. Regional Poultry Research Laboratory, East Lansing, Mich.

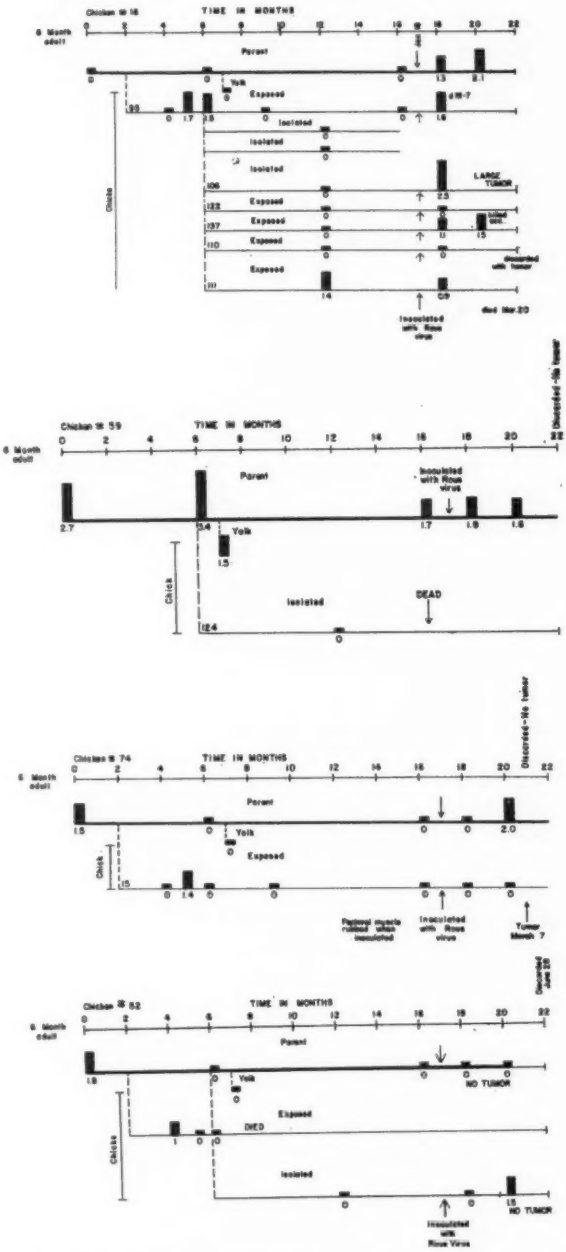
†Courtesy Dr. S. Kenzy, of College of Veterinary Medicine, State College of Washington, Pullman, Wash.

Prevalence of Antibody

The emphasis in our study has been on the life history of individual chickens, so that relatively little has been done on the prevalence of anti-



TEXT-FIGURE 1.—Neutralizing antibodies in sera from individual chickens during 22 months observation. The figures under the *bars* refer to the neutralization titer



of the serum expressed as the calculated log dilution 10 of the serum which reduced 10 ID50's to the 50 percent end point.

body. However, in table 3 it may be seen that about half of the 6-month-old chickens had antibody but that only 1 of 15 chickens, 18 months or older, had such antibodies. These results have more meaning in relation to the individual histories that are graphically presented in text-figure 1. Of the 8 chickens, 5 had antibodies when the study started, and 4 of the 5 lost their antibody during the next 6 months. This was at first thought to be due to some technical error or a change in the virus. However, the loss of antibody titer was confirmed in chicks in which the earlier sera were tested simultaneously with the second negative sera. None of the 4 chickens regained antibody during the next year of observation, nor did the 3 chickens which at the start had lacked antibody. One chicken maintained a high titer of antibody throughout the period of study. The rooster (not included in text-fig. 1) lacked antibody at the start of the experiment and at no time developed a significant titer. As shown in text-figure 1, however, the lack of antibody did not necessarily depend on an inherent inability to develop antibody, for 2 of these negative chickens developed significant antibody when inoculated with Rous virus.

TABLE 3.—*Neutralizing antibodies in White Leghorn flock—random sera acquired at different ages*

Age (*months*)	No. tested	No. with neutralization as expressed in log units neutralized			
		0–1	1–1.4	1.5–2.4	2.5+
6	9	4	0	3	2
18	10	8	1	1	0
24	5	4	1	0	0

Elsewhere we have noted that tissue cultures of about 15 percent of chick embryos obtained from this flock of chickens yield large numbers of particles that are associated with destroyed cells (*14*) and indistinguishable from Rous virus. For this reason our original experimental grouping, in which half of the hatched chicks were separated from their parents and the other half kept with the parents, was again subdivided. One half of each new group comprised chicks from hens with antibodies to Rous virus, and the other half, chicks from hens without antibody. There was no apparent effect of the neutralizing titer of the parent hen. However, as may be seen in table 4, only those chicks that were in some degree

TABLE 4.—*The development of neutralizing antibodies to Rous virus dependent on exposure to adult chickens*

	Total number	Highest neutralization titer acquired		
		0–0.9	1.0–1.4	1.5+
Exposed	12	5	4	3
Isolated	8	8	0	0

exposed to the "infected" parents acquired antibody. Of these 12 chicks, 3 developed significant antibody and 4 an equivocal titer. None of the 8 isolated chicks developed antibodies.

The study of this flock was concluded by injecting an undiluted suspension of Rous virus into the pectoral muscles of all the chickens. In the 12 chickens that survived long enough for observation of tumor development, there was no apparent relationship between the past history of antibody and the development of tumors.

Discussion

Little is known about the spread of Rous virus through a population of chickens (*12*). Shrigley (*15*) has raised the possibility that the virus spreads by the respiratory route, for intranasal inoculation of chicks in his studies was followed not only by pulmonary tumors but also by the development of tumors elsewhere. In our experiments no attempt was made to determine the method of spread within the population, but it is apparent that it occurred at an early age.

Populations of chickens, although belonging to one "breed," are well recognized as genetically heterozygous. When, in addition, it is remembered that there are stocks of chickens resistant to the Rous virus (*16*), it was not surprising to find some chickens that apparently did not acquire the infection. Three of the 16 chickens in our flock failed to develop antibody even when Rous virus was inoculated into their muscles. It will be interesting to study the mechanism of this resistance in more detail.

The common result in our flock was that the chick developed antibodies within 2 to 6 months, and then lost them during the following 6 to 12 months. Some of these chickens, however, were able to produce antibodies again when inoculated by Rous virus as shown by such examples as #74 and #59 (text-fig. 1). The disappearance of neutralizing antibody seems at first unusual, but Buescher (*17*) has reported a similar rise and fall in antibody against Japanese B encephalitis virus following infection of the chick. Furthermore, Buxton has shown that antibody is suppressed in chicks that have received large doses of antigen early in life (*18*). The possible presence of virus in the egg and developing embryo may then influence the final antibody response of the host.

The studies on neutralization of Rous virus activity with the production of a tumor as the end point have been extensively summarized (*6*). In addition, Kenzy has used a rough quantitation of this neutralization to confirm the presence of antibodies (*2*) against Rous virus in normal chickens, which are particularly prevalent if lymphomatosis is endemic in the flock (*19*). However, when a strain of lymphomatosis was transmitted through passage in other chickens the incidence of antibodies to Rous virus dropped (*20*). Chickens raised in isolation failed to develop antibodies (0/70), whereas other chicks of the same stock raised with lymphomatous chickens developed a high incidence of antibodies (15/16), (*21*). Recent antibody studies by Beard *et al.* indicate a relationship between lymphomatosis and Rous virus (*13*). Thus, at the present time, it is

not established whether the development of antibodies to Rous virus occurs solely in response to that virus or whether there is some overlap with other tumor viruses. This study was undertaken in the belief that the measurement of antibody to the destructive effects of the virus might yield more specific data, which would more definitely measure virus antibodies, and not measure antibody to the cells or other non-specific neutralizing substances.

In several respects, however, the present data confirm some of Kenzy's findings. Antibody did not appear in isolated chicks and it disappeared from the serum of some adult chickens (*19*). The final determination of the relationship of these viruses will, however, depend on a clear separation of antibody to cells from antibody to the virus (*4*), a separation which should now be possible with the several recent refinements in quantitative measurements of virus activity (*22–24*). Regardless of the eventual relationship of Rous virus to lymphomatosis, it will remain of extreme interest to determine the significance of the antibody response in relation to the occurrence of tumors in a natural flock, which, according to Foulds, frequently occur early in adult life (*25*).

Summary

1) Antibody to the Rous sarcoma virus was measured by determining the dilution of serum that neutralized the ability of the virus to produce hemorrhagic lesions when injected intravenously into chick embryos.

2) Of nine 6-month-old chickens (1 rooster), 5 had antibodies, but showed no spontaneous tumor. Of these 5 "positive" birds, 4 lost their antibody during the next 6 months, and did not "spontaneously" regain their antibody in the next year. The disappearance of antibody was also found in an independent sampling of a group of chickens 1½ to 2 years of age.

3) Individual chicks derived from this flock were continuously observed during the same period of time. None of them developed tumors. Antibody was found in the yolk of the eggs from which they had hatched and presumably was absorbed by the developing chicks. Of 12 chicks kept with their infected parents, 3 developed significant antibody by 6 months of age, and 4 an equivocal titer. None of these maintained their titer. None of the 8 isolated chicks developed antibody to Rous virus. There was no direct or inverse relation between the presence of the antibody in the hen (and thus presumably in the yolk) and the subsequent acquisition of antibody by the chicks.

4) At the conclusion of the experiment, all the chickens were injected with Rous virus. Several chicks that had lost their antibody regained antibody within 1 to 2 months. The series was too small to determine whether there was a relation between the development of tumor and the past history of antibody.

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Virus of Avian Myeloblastosis. X. Photometric Microdetermination of Adenosinetriphosphatase Activity [1,2,3]

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Blood plasmas from chickens diseased with myeloblastosis (*1*, *2*) act strongly to dephosphorylate adenosine triphosphate (*3*). The activity is a property of the filterable agent specifically responsible for the disease and is quantitatively related to virus particle number (*4–6*). In consequence, the enzymatic behavior, under controlled conditions, constitutes a biochemical criterion for measurement of virus concentration and amount in terms of number of virus particles. The enzyme activity can be measured accurately by conventional methods for estimation of the inorganic phosphorus liberated from adenosine triphosphate (*7*). Such procedures, however, are not adaptable to the numerous possibilities for investigation of this agent and its host cell. A procedure for measurement of the acid liberated in the hydrolysis of adenosine triphosphate by the virus in minute volumes of plasma has been very useful (*8*) in the rapid screening of large numbers of chicks for high virus concentration in the plasma. This method, employing the rate of change in the color of an indicator system, has exhibited many deficiences that make it unfit for consistent quantitative work over protracted periods. Further study during the past 2 years has resulted in the development of a different reaction system based on the same principle. Utilizing a color change estimated photometrically, the procedure offers the advantages of small volumes of test materials; accuracy, as checked by repeated virus particle counts; and reproducibility and control of the reaction system. The method, which has been used in tissue-culture studies of another report (*9*), is described in the present paper.

Materials and Methods

Stock solutions for preparation of the final reaction mixture (substrate-indicator solution) were NaCl, 0.5 *M*; $MgCl_2$, 0.5 *M*; $NaHCO_3$, 0.06 *M*;

[1] Received for publication July 29, 1957.

[2] This work was supported by a research grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society, Inc., on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

[3] In earlier publications from this laboratory, the disease under study was designated erroneously as "erythromyeloblastosis." The correct term is "myeloblastosis," which will be employed here and in further reports of studies on this virus.

and phenol red, 1.41 m*M*. The salt solutions were kept in pyrex bottles at room temperature, and the phenol-red solution was stored in a like vessel at 2° C. A solution of 0.2 *N* NaOH, used for adjustment of *p*H, could be kept as a stock solution if rigid precautions were taken to prevent absorption of CO_2; otherwise, the base must be prepared frequently, since enough CO_2 could be taken up on long standing to affect the reaction rates.

The *substrate* was crystalline disodium adenosine triphosphate (Na_2 ATP·$3H_2O$) obtained from Sigma Chemical Co., St. Louis, Missouri. It was kept in the original shipping bottle over silica gel in a tightly closed jar at −20° C.

Standard enzyme (virus) for controlling the activity of the reaction mixtures was plasma from individual chickens diseased with myeloblastosis. The plasma was diluted 1:10 with Gey's salt solution (*10*) and stored in 0.5 ml. volumes in 1 ml. glass-sealed vials at −20° C. As one lot of standard was depleted, another was prepared and thoroughly checked for level of activity.

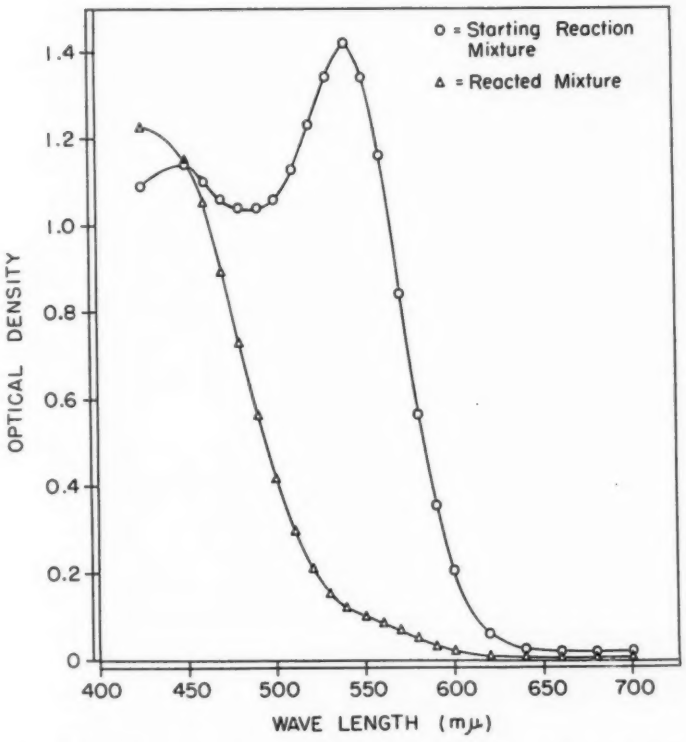
A *color standard* consisted of 10 ml. of the phenol-red stock solution diluted to 100 ml. with 0.1 *M* phosphate buffer of *p*H 7.5. It was kept in a rubber-stoppered pyrex flask at room temperature.

For preparation of the substrate-indicator solution, 10.0 ml. of each of the NaCl and phenol-red stock solutions were added to 300 mg. of adenosine triphosphate. The volume was brought to about 70 ml. with deionized water, and the adenosine triphosphate was dissolved with the aid of a magnetic stirrer.

While the stirring was continued, 8.0 ml. of $MgCl_2$ stock solution were introduced, and the *p*H was adjusted to about neutral with the 0.2 *N* NaOH. Finally, 0.8 ml. of $NaHCO_3$ stock solution was added; the *p*H was brought to 7.5 with NaOH; and the total volume was increased with water to 100 ml. This substrate-indicator solution was stored in 50 ml. lots at −20° C. The solution was useful for several days under these conditions. It is notable that the final concentrations of the salts and the *p*H were optimal for the activity of the enzyme as determined in earlier studies (*3*).

Determination of enzyme activity was made by measurement of the rate of change in the optical density of the substrate-indicator solution under the influence of the phosphoric acid liberated. The reaction was followed with the Coleman, Jr., spectrophotometer at a wave length of 550 mμ, which afforded a favorable difference between the starting optical density of the reagent at *p*H 7.5 and that at the acid or reacted *p*H as shown by the respective absorption spectra in text-figure 1.

For the determination, the spectrophotometer was set at 0 transmittance in the usual way and then readjusted to 0 optical density with a water blank. Check of the color standard gave a value of about 1.25 optical density; the instrument was then adjusted to indicate an optical density of .500 for the color standard. A volume of 1.0 ml. of the substrate-indicator solution in a Coleman tube (10 × 75 mm.) was checked against



TEXT-FIGURE 1.—Absorption spectra of the substrate-indicator solution before addition of enzyme (virus) and of the mixture at conclusion of the reaction of the virus.

the color standard reading of .500. To this was added and thoroughly mixed, 50λ of an appropriate dilution of the virus (enzyme) preparation to be tested. The tube was returned to the instrument and the optical density was recorded. With progress of the reaction, the optical density decreased. The rate of reaction was the time in seconds required for decline in optical density through the region from .350 to .300. This corresponded to a *p*H change from about 7.29 to 7.26.

The activity of the substrate-indicator solution was controlled by checking against the standard virus preparation before starting with the unknown test materials and at suitable intervals during a given series of assays. With the values yielded by the standard enzyme, corrections were made, if necessary, by multiplication of the reaction rates of the samples by a correction factor which was the ratio of the initially determined standard time to the time observed with the standard on the day of the assay. The determinations were made at room temperature which varied from 22 to 27° C.

Characteristics of the Assay

Variation in substrate activity.—The principal difficulties in establishing a procedure for assay of the enzyme activity based on measurement of the rate of acid liberation have been related to variations in the response of the substrate solutions to the action of the virus. It was for this reason that the potentiometric methods used earlier (*3*, *4*) have been abandoned and recourse made to the more time-consuming but accurate procedures for determination of liberated inorganic phosphorus (*7*, *11*). Especially troublesome has been the substrate system employed in the microscreening procedure, which, although consistent in activity under limited conditions (*8*), may yield results of wide variation from day to day and from one batch of reagent to another. Despite the difficulties, the microscreening method has been retained in the form originally described and controlled by checks with a standard plasma in simultaneous examinations with unknown samples.

Many improvements have been attained in the present system. Measurements can be made more rapidly than by the determination of phosphorus and, in consequence, the procedure can be applied to the large numbers of samples essential for many studies such as those involving tissue culture (*9*) of the virus. The results are of relatively high absolute accuracy, since the reactivity of the substrate solution can be closely controlled by routine references to a stable standard. Further check is available by counts of the virus particles of the standard and of unknown preparations (*9*).

Correction of reaction time.—It has not yet been possible to discover all the factors influencing or the means for elimination of variation in the reactivity of substrate-indicator solution. With care, however, this can be reduced to a narrow excursion, and appropriate corrections against the standard values yield very satisfactory data. An example of the results obtained in repeated studies on a series of 6 samples of tissue-culture fluids examined on different days is shown in table 1. Here, it is seen that the reaction time with the standard, which was initially found to be 18 seconds, varied from a low of 15 to a high of 23 seconds. Application of the correction factor to the values obtained by simultaneous examination of the unknown samples yielded corrected reaction times of remarkably little variation.

Variation during 1 day.—The results of repeated assays on 2 different samples of the virus enzyme on the same day are shown in table 2. In 10 assays on each, the respective coefficients of variation were 4.2 and 2.8. These values are favorably comparable with the results obtained with other methods for study of the enzyme.

Variation over an extended period.—Four virus plasmas were diluted tenfold with Ca-free Ringer's solution; parceled in 0.5 ml. aliquots; and stored in 1 ml. glass-sealed ampoules in a CO_2 chest at −78° C. Virus samples were removed at the times indicated in table 3 and assayed. The measurements were made in triplicate on each plasma, and the mean values, corrected for the standard after the method described in an earlier

TABLE 1.—*Application of correction factor to assay values obtained at different times on 6 samples of tissue-culture fluid*

Sample	Date	Standard time* on assay date (*sec.*)	Sample reaction time (*sec.*)	Corrected† reaction time (*sec.*)
1	12/ 7/56	15. 0	33. 0	39. 6
	12/27/56	16. 5	36. 8	40. 1
2	12/ 7/56	15. 0	20. 8	25. 0
	12/27/56	16. 5	24. 5	26. 7
3	12/ 7/56	15. 0	11. 2	13. 4
	12/27/56	16. 5	13. 2	14. 4
	12/28/56	23. 0	17. 0	13. 3
4	12/ 9/56	16. 0	20. 6	23. 2
	12/ 9/56	16. 0	20. 2	22. 7
	12/ 9/56	16. 0	20. 6	23. 2
	12/27/56	16. 5	21. 0	22. 9
5	12/11/56	19. 6	53. 0	48. 7
	12/27/56	16. 5	46. 5	50. 8
6	12/27/56	16. 5	136. 0	148. 0
	1/ 3/57	19. 8	162. 0	147. 0

*Standard reaction time=18 seconds.

†$\frac{\text{Standard reaction time}}{\text{Reaction time of standard on day of assay}} \times$ sample reaction time.

TABLE 2.—*Results of repeated observations on 2 samples on the same day*

Reaction time (*sec.*)	
Sample 1	Sample 2
24. 0	104. 5
22. 8	110. 8
23. 0	103. 6
22. 5	105. 4
23. 6	107. 4
23. 6	111. 0
23. 0	108. 8
21. 0	110. 4
22. 0	112. 0
21. 2	111. 8
22. 7*	108. 6*
0. 92†	2. 98†
4. 2‡	2. 8‡

*Mean.
†Standard deviation.
‡Coefficient of variation.

section, are recorded in the table. The mean coefficient of variation of the values obtained with the 4 plasmas was 10.8. There was an apparent increase in assay value, which may have been a reflection of variation of the correction standard.

TABLE 3.—*Repeated measurements on 4 standard plasma preparations over extended period*

Date of assay	Plasma reaction time (*sec.*)*			
	G945	G985	G982	G989
10/22/56	58. 6	9. 3	113	11. 2
10/25/56	63. 7	10. 5	139	11. 7
11/7/56	57. 4	9. 3	113	11. 1
12/1/56	61. 4	9. 9	135	11. 6
2/11/57	74. 7	11. 9	166	13. 5
2/11/57	75. 6	12. 0	158	12. 5
4/12/57	75. 2	12. 3	149	12. 7
Mean	66. 7	10. 7	139	12. 0
Standard deviation	7. 60	1. 22	19. 1	0. 82
Coefficient of variation	11. 4	11. 4	13. 7	6. 8

*Corrected.

The following events transpired during the course of the assay period: 1) the standard enzyme was changed four times; 2) two separate lots of adenosine triphosphate were used for preparation of the substrate-indicator solution; 3) three different people were involved in collection of the data; and 4) the substrate-indicator solution was modified by the addition of sodium bicarbonate. In general, this test of the reliability of the assay demonstrates that the data can be reproduced quite well over a 2-month period and that only a small variation occurs over an interval of 6 months, some of which could have resulted from experience in the development of the procedure.

Variation with different lots of adenosine triphosphate.—Only 2 lots of adenosine triphosphate, purchased at different times, have been used in the assays. The results with substrate preparations of each are shown in table 4. For these comparisons, the same plasmas and standard preparations described in the preceding section were employed (table 3). The differences between the 2 lots were not significant, since they were well within the limits of variation observed with the individual substrate preparations from the same lot of adenosine triphosphate.

Optical density and the stage of reaction.—The experimental basis for choice of the range of change in optical density from .350 to .300 for measurement of the rate of activity is illustrated in text-figure 2. Here it is seen that optical density is not linear throughout the entire course of the reaction. A linear relationship was observed, however, from above .350 to well below .300. The time sequence thus afforded opportunity for preparation of the sample, its introduction into the instrument, and a satisfactory region of linear change for determination of the rate of reaction.

Relation of reaction rate to sample dilution.—It was shown in earlier studies (*3*) that, within the limits of the methods of assay, enzymatic activity was proportional to virus amount and independent of the influence

TABLE 4.—*Comparison of enzymatic activity of 4 plasmas with substrate-indicator solution prepared from 2 different lots of adenosine triphosphate*

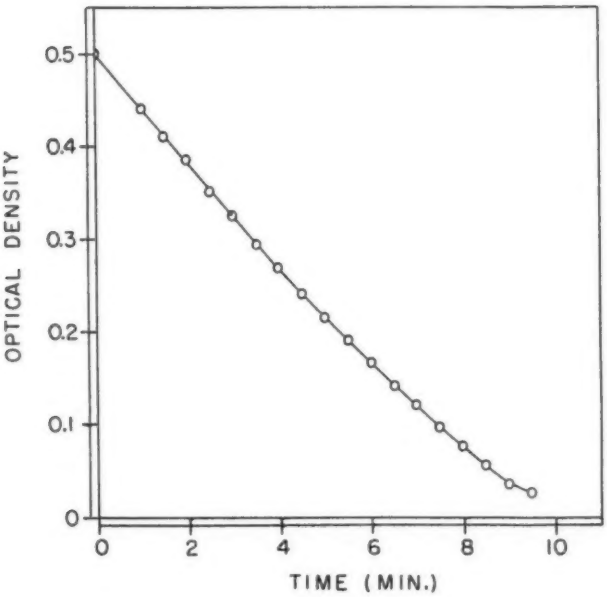
Sample	Reaction time (*sec.*)*			
	Lot #66–91	Mean	Lot #126–91	Mean
G945	74. 6 77. 6 74. 8		74. 5 74. 5 75. 1	
		75. 7		74. 7
G982	175. 8 144. 2 155. 5		173. 7 157. 3 169. 0	
		158. 5		166. 6
G989	12. 2 12. 4 12. 9		13. 5 13. 8 13. 3	
		12. 5		13. 5
G985	12. 2 11. 5 12. 4		11. 9 11. 9 11. 9	
		12. 0		11. 9
Standard P191	22. 0		23. 9	
Standard L88	20. 6		23. 0	

*Corrected values.

of dilution *per se*. Similar results were observed in the present experiments. Examples of the findings in these studies are seen in text-figure 3. Specimens of two plasmas were diluted with Gey's salt solution to contain the volumes of plasma indicated in the abscissa in the 50 λ samples added to the reaction mixture. In both instances, the reciprocal of the reaction time was linear with plasma volume through the range of 0.36 to 12.5 λ of plasma.

Influence of ammonia on the assay.—The procedures described in this report have been utilized most intensively thus far for assay of the virus in tissue-culture fluids (*9*). Many samples of such materials are slightly acid, and adjustment of *p*H is essential before the reaction rate can be determined. This can be accomplished without dilution of the sample by the introduction of small quantities of ammonia gas. That the presence of ammonium salts in such small concentrations did not influence the assay is revealed in table 5.

For this study, 1 ml. of virus plasma was diluted with 19 ml. of Gey's salt solution, with a resulting *p*H of 7.64. A sample was removed; the remaining solution was acidified with HCl gas to successively lower H^+ concentrations as recorded in the table; and samples were removed at each level. The individual samples were readjusted to the original *p*H



TEXT-FIGURE 2.—Optical density of the virus-substrate indicator solution mixture with progress of the reaction.

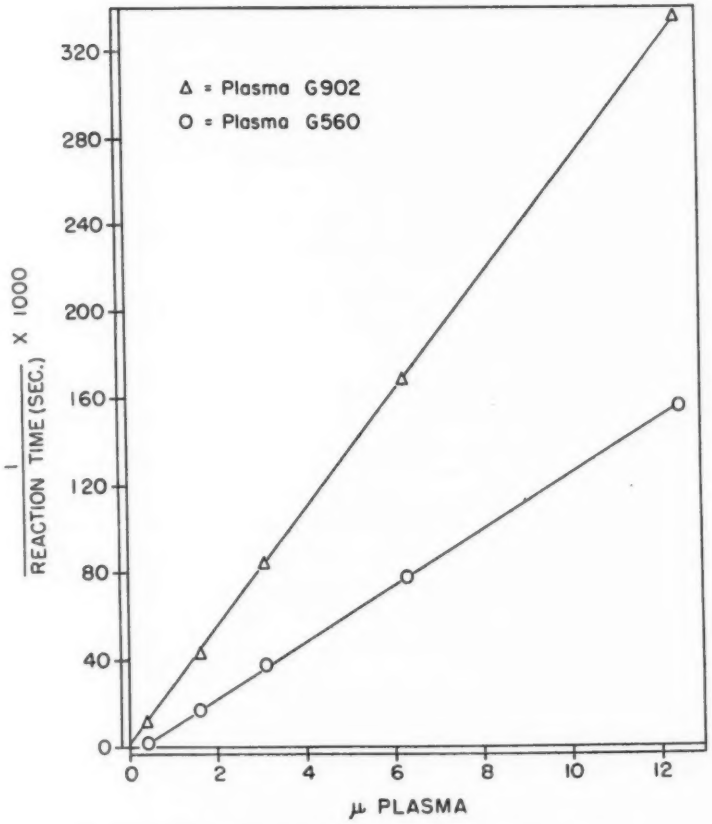
TABLE 5.—*Effect of ammonia on rate of reaction*

*p*H before adjustment	Reaction time (*sec.*)			
	Triplicate assays			Mean
7.65 (no adjustment)	24. 8	25. 3	26. 4	25. 5
6.80	25. 3	25. 7	26. 0	25. 7
6.55	25. 6	26. 0	25. 3	25. 6
6.11	28. 0	27. 1	25. 1	26. 7

with NH_3 gas, and assayed for enzyme activity. The mean values of the neutralized samples did not vary significantly from the result obtained with the control.

Discussion

The new procedure described here has provided a major improvement over the previously employed method (*8*) for the rapid and accurate assay of the adenosinetriphosphatase activity of the avian myeloblastosis virus. This has been due in large part to the uniformity of activity and to the stability of the substrate-indicator solution and, as well, to the use of standard preparations of the enzyme (virus) which is of high stability at −78° C. Inasmuch as the enzyme activity is quantitatively related to the virus particle, the results observed, when correlated with particle



TEXT-FIGURE 3.—Relation of reaction time to plasma volume.

count, constitute a measure of *absolute* virus amount or concentration. The reaction solution containing salts in optimal concentrations, phenol red as indicator, and adenosine triphosphate as substrate represents the most stable and least variable system thus far devised for the purpose. It has not been possible yet to distinguish factors responsible for the small variations remaining, but a large degree of control is possible by frequent reference to stable standards.

It should be emphasized that the range of variation must be considered from two points of view. As a method for chemical assay, the results may be regarded as rather variable. On the contrary, however, as a means for assay of virus amount or concentration, the findings exhibit high precision. Bioassay of the virus in the chick host is time consuming and expensive, and the results, even with very large numbers of chicks (*12*), are too variable for the quantitative data demanded by some fields of study. Many investigations, including those involving tissue culture to be described in

another paper (*9*), would be impractical, if not quantitatively uninformative or misleading, with access only to infectivity tests.

Summary

A photometric micromethod has been developed and applied to the measurement of the adenosinetriphosphatase activity associated with the virus of avian myeloblastosis. The method is based on the rate of change in the optical density of a substrate-indicator solution following increase in hydrogen-ion concentration which is proportional to the liberation of the terminal phosphate of adenosine triphosphate. The substrate-indicator solution consists of adenosine triphosphate together with sodium and magnesium ions in optimum concentration and phenol red. Repeated measurements on a given day gave highly uniform data, the average coefficient of variation being about 3, which was approximately that to be expected by direct determination of phosphorus. Greater variation was observed from one day to another due to factors yet unknown. Very satisfactory control could be effected, however, by continual standardization against stable control preparations of the virus, a practice which yielded results of an average coefficient of variation of 10.8 over a period of 6 months. Since enzyme activity is quantitatively related to the virus, the procedure constitutes a convenient and rapid means for assay of the agent not only in investigations with the chicken but particularly with those encountered in the multitude of opportunities for study of cell-virus relationships in tissue culture.

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Virus of Avian Myeloblastosis. XI. Release of the Virus by Myeloblasts in Tissue Culture [1,2]

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A major manifestation of the virus-induced avian leukemia, myeloblastosis (*1*, *2*), is the occurrence in the circulating blood of primitive cells in numbers varying to levels of 2 million per mm,[3] (*3*). Studies have been made of these cells in tissue culture. It has been found that the myeloblasts can be maintained for long periods *in vitro* and that the myeloblastosis virus is continually liberated into the culture fluid. The results of investigations on various aspects of the phenomenon are described in the present report.

Materials and Methods

Myeloblastosis.—The disease under study was the avian myeloblastosis (*2*, *4*, *5*), designated as BAI strain A, derived originally by Hall, Bean, and Pollard (*6*) and observed in this laboratory since 1949 (*7*). Because of former uncertainty of the etiologic relationship (*8–10*) between the two principal forms of avian leukemia, myeloblastosis and erythroblastosis, the agent now recognized as that of myeloblastosis was designated erroneously in earlier publications as the virus of erythromyeloblastosis. From recent studies (*2*, *4*, *5*) it has been established that myeloblastosis and erythroblastosis are not only distinct pathologically (*1*, *11*), but that they are caused by entirely different, though related (*10*), viral entities. For this reason, the term "erythromyeloblastosis virus" has been discontinued, and it is to be emphasized that *all properties previously ascribed to the virus of erythromyeloblastosis, as far as this laboratory is concerned, were actually those pertaining to the agent of myeloblastosis.*

Myeloblasts for culture.—Donors of cells and virus were inbred White Leghorn chicks of line 15 (*12*, *13*) of the Regional Poultry Research Laboratory, East Lansing, Michigan. Some of the donors were chicks inoculated with virus filtrates at 3 to 5 days of age (*14*), but more often injections were made into 10-day-old birds, which also develped high concentrations of both cells and virus in the circulation. Donor selection

[1] Received for publication July 29, 1957.

[2] This work was aided by grants to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

was based on blood smears showing large numbers of the primitive cells and on adenosinetriphosphatase activity (*15*) for high concentration of virus in the plasma. The birds were bled in the usual way and the cells and plasma were separated by centrifugation.

The loosely packed white-cell layer, usually about 2 to 3 ml. in volume, was transferred by pipetting to a 15 ml. graduated centrifuge tube, and the cells were resuspended in 10 ml. of Gey's salt solution (*16*). Washing was carried out by 3 or 4 repeated sedimentations of the cells from 10 ml. volumes of the salt solution. Aliquots of the cells packed in the last sedimentation were taken up in the desired culture medium in concentrations of 0.05 to 0.1 ml. packed cells per 5 ml. of culture fluid. These concentrations corresponded to approximately 2.5 to 5 $\times$ 10^7 cells per ml. of medium.

The suspended cells in 5 ml. volumes were cultured in 50 ml. Erlenmeyer flasks closed with rubber stoppers. These were incubated at 37° C. on a rotary shaker at about 70 to 80 cycles per minute (Gyrotory Shaker, S3, New Brunswick Scientific Co., New Brunswick, N. J.). Representative samples of the cultures were removed at "zero time" and at indicated intervals for cell counts, assay of enzyme activity, particle counts, and, in some instances, for titration of infectivity. The tissue-culture medium was changed at 1- to 6-day intervals. The whole cell suspension was transferred to a 15 ml. graduated centrifuge tube, and the cells were spun down at about 150 $\times$ g for 10 minutes. The supernatant fluid was removed and stored at −20° C. for enzyme assay and particle counts. Materials to be tested for infectivity were stored at −78° C. After resuspension in fresh medium, the cells were returned to the *original* 50 ml. Erlenmeyer flask.

Virus assay—Enzymatic activity.—Routine determination of virus amount was made by measurement of adenosinetriphosphatase activity with the method described in an accompanying paper (*17*). A reaction time of 1,000 seconds for a 50 λ sample of culture fluid was selected as one unit of enzyme activity. This was found (*17*) to correspond, on the average, to 4.57 $\times$ 10^7 virus particles per 50 λ volume or, as employed for computations, 9.15 $\times$ 10^8 particles *per ml.* of test preparation. *Unless otherwise indicated, particle numbers cited were those determined by enzyme measurement.*

Particle count.—Direct determinations of particle concentration were made (*18*, *19*) by sedimentation of the virus onto an agar surface and electron microscopic examination of pseudoreplicas of the particles formed with collodion and shadowed with metal. The volume of blood plasma needed was 1 λ and that of culture fluids was 10 λ. These quantities were diluted to 1.0 ml. for sedimentation.

Titration.—Virus infectivity of the various preparations was assayed with the latent period procedures (*7*, *20*) devised for this virus. Control preparations consisted of dilutions of a standard pool of myeloblastosis virus kept under crushed CO_2 ice (−78° C.). With few exceptions, as will be noted, the preparations tested for infectivity were *filtered* before use.

Culture medium.—The constituents of the culture medium were Gey's salt solution (*16*), antibiotics (50 units of penicillin and 50 mg. of streptomycin per ml. of medium), normal chicken serum, and added glucose. The proportions of the various materials are described with the respective experiments. Chicken serum was obtained from normal birds of line 15, most of which were 3 to 6 months of age and some as old as 1 year; they were fasted for 24 hours before bleeding. All serums were heated at 56° C. for 30 minutes immediately before use in culture medium.

Experiments and Results

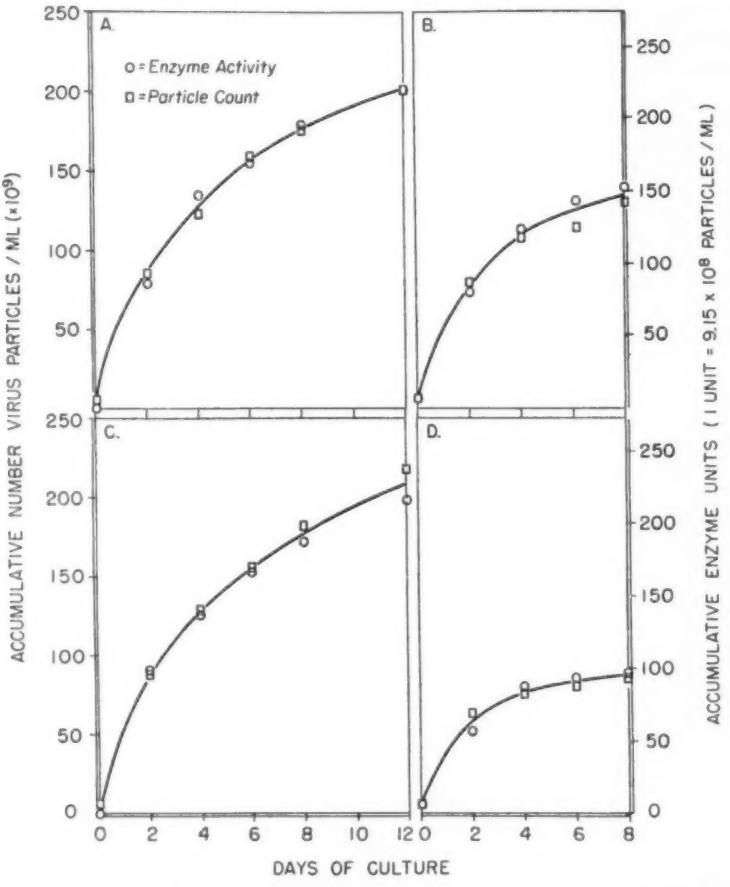
1. Correlation of adenosinetriphosphatase activity with particle count.—Adenosinetriphosphatase activity has been closely related to virus particle number in the plasmas and ultracentrifugal concentrates of the plasmas from birds with myeloblastosis (*3*, *19*, *21*, *22*). The only independent means for quantitative estimate of absolute virus amount in this disease is that of particle enumeration. Because of the dependence on enzyme measurements for routine estimates of virus particle number in the present experiments, studies were made of the relationship between particles counted directly and enzyme activity determined under the conditions of the tissue-culture experiments. An illustration of the findings is provided by the results obtained with 4 groups of cultures, as shown graphically in text-figures 1A–1D.

In the experiment of text-figures 1A and 1C (expt. 5556) myeloblasts were obtained from a single bird (L 117), and 6 cultures containing about 6.7×10^7 cells per ml. were prepared. The medium was *20 percent* normal chicken serum in Gey's salt solution containing 0.5 percent glucose and antibiotics. The tissue-culture fluids from 3 of the flasks were pooled at the time of each fluid change (text-fig. 1A) and those from the other 3 flasks were similarly treated (text-fig. 1C) at the same intervals. Each pool of the 2 sets of cultures was assayed for enzyme activity and particle count. Text-figures 1B and 1D (expt. 5256) show the results of similar studies on 6 cultures of cells from a different bird (L 113) in a medium containing *5 percent* normal chicken serum and 0.1 percent glucose.

In the graphs it is seen that accumulated adenosinetriphosphatase activity, expressed in terms of enzyme units, was very closely related to the accumulated number of virus particles determined by direct count.

2. Medium factors influencing virus liberation.—As already indicated in text-figures 1A–1D, culture of the myeloblasts *in vitro* was attended by liberation of the agent into the culture fluid. Gey's salt solution, containing chicken serum and added glucose, provided a medium optional for maintenance of the cells and with virus liberation for periods of at least 6 months.

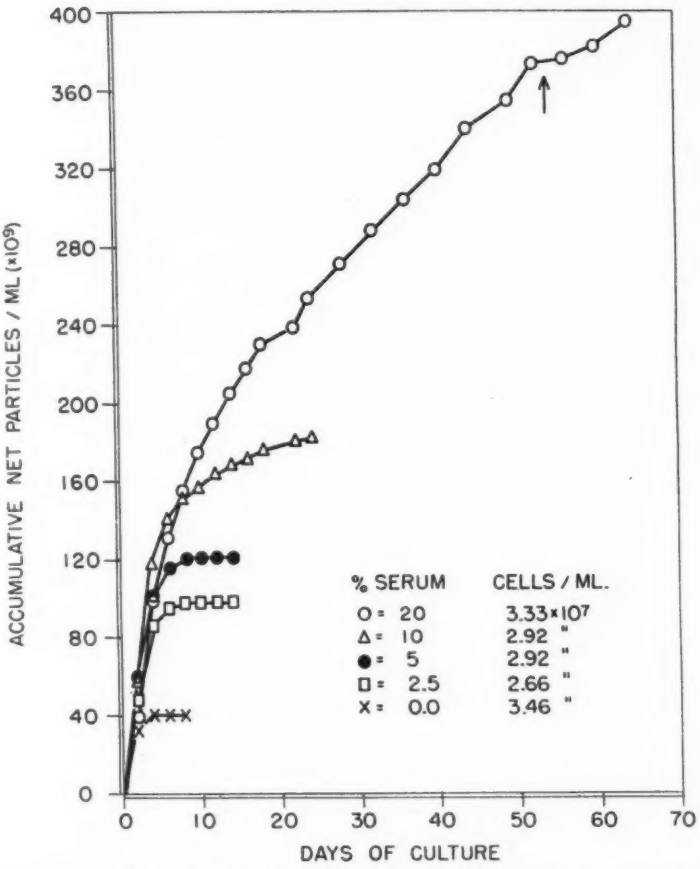
Chicken serum in a relatively high concentration was essential for progressive virus liberation. The relationship is illustrated by the results of a typical experiment as seen in text-figure 2 (expt. 5456). A series of 5 pairs of cultures was established with the cells from bird L 146. Each pair contained a different concentration of serum ranging from 0 to 20 per-



TEXT-FIGURE 1.—Relationship of adenosinetriphosphatase activity of tissue-culture fluids to the number of virus particles in the fluids determined by direct count.

cent and 1.0 percent added glucose. The rate of virus liberation is plotted as the mean value for each pair of cultures. At the level of 20 percent serum, virus release has continued for at least 6 months (*see* text-fig. 3). Glucose, likewise, was a necessary component of the medium. The data observed in 3 experiments are given in table 1. For experiment 4756, 4 cultures of cells from one bird were set up at each of the different concentrations of glucose. In experiments 4856 and 4956, the cultures at each glucose concentration were prepared in pairs, and each value in the table is the mean found with each pair. Chicken serum was present in 5 percent concentration.

In the absence of *added* glucose, virus was found in the 1-day fluids but in amounts less than those in the cultures containing extra glucose. In these cultures no virus was liberated after the first day. Concentra-

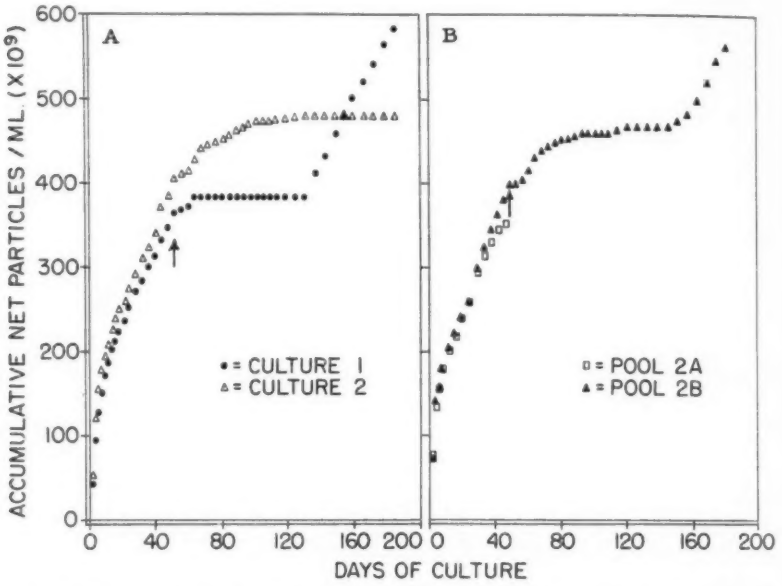


TEXT-FIGURE 2.—Influence of the concentration of chicken serum on the rate and continuance of virus liberation by myeloblasts in tissue culture.

tions of glucose in the range between 0.1 and 1.0 percent were equally effective in supporting virus release.

3. Rate of virus liberation.—Initial rates of virus release were always greater than those seen in older cultures. The findings within the first 48 hours are illustrated in table 2 with the data of 3 experiments with 18 separate cultures. Each experiment was made with the cells from one bird. The cultures contained chicken serum in 5 or 20 percent concentration and 0.1 percent or greater concentration of glucose. Analyses of the results obtained in 9 experiments with 38 cultures gave a mean rate of 31.2 $\pm$ 9.4 particles released per cell per hour.

Subsequent rates observed with cells kept for a total period of 12 days in medium containing 20 percent chicken serum and 1 percent glucose have been shown in text-figures 1A and 1C. The findings with cells maintained for longer intervals are seen in text-figures 3A and 3B.



TEXT-FIGURE 3.—Course of virus liberation by cultures of myeloblasts carried for 6 months. The *arrows* in both charts indicate the point of use of serum 856 (*see* text-figs. 2 and 3) which caused decrease in the rates of virus release in all cultures receiving it.

In text-figure 3A are the results of an experiment (5456) with 2 individual cultures of cells from bird L 146. Initially, culture 1 contained 3.3×10^7 cells per ml., and the concentration of the cells in culture 2 was 2.9×10^7. In both, the medium was 20 percent chicken serum with 1.0 percent glucose for the first 52 days. The concentration of glucose was 0.5 percent thereafter. Fluid changes were made at 2-day intervals for the first 18 days, at 4-day intervals until the 120th day, and then at 6-day intervals. Both cultures are still intact as indicated.

Experiment 5556, text-figure 3B, comprised 6 cultures of cells from bird L 117. The cell concentration was 6.67×10^7 per ml., and the medium contained 20 percent serum and 0.5 percent glucose. The cultures were started as 2 groups of 3 each, series 2A and 2B, but analyses were made on each fluid separately. In the graph of text-figure 3B, each value charted is the mean obtained for the respective groups until the 34th day. At that time 1 culture of series 2A was lost, and another was discontinued after 38 days. The 3rd culture of series 2A was discarded at 50 days. Of series 2B, 1 culture was discontinued at 61 days and another at 85 days. After this time, the data observed with series 2B were those concerned with a single culture.

At the fluid change indicated by the *arrows* in both figures (*see* also text-fig. 2), serum of lot 856 (table 3) was used. There was a marked

TABLE 1.—*Accumulative net virus release per ml. ($\times 10^9$) for various concentrations of glucose in the culture medium*

Culture period (*days*)	Percent glucose			
	0	0.1	0.5	1.0
Experiment 4756				
1	12. 3	13. 5	16. 3	15. 6
2	14. 2	32. 3	32. 8	30. 9
3	14. 4	50. 0	54. 5	52. 6
6	14. 4	98. 3	124. 3	108. 7
9	14. 4	128. 6	146. 9	126. 4
Experiment 4856				
1	26. 7	37. 3	43. 7	40. 4
2	26. 6	55. 4	60. 8	65. 1
3	21. 4	60. 4	59. 9	87. 8
6	21. 4	74. 8	74. 3	114. 7
9	21. 4	76. 6	75. 9	120. 1
Experiment 4956				
1	16. 8	26. 2	30. 0	25. 0
2	20. 7	47. 0	45. 1	41. 1
3	20. 0	60. 1	52. 9	52. 1
6	20. 0	85. 4	74. 1	67. 5
10	20. 0	94. 6	84. 4	74. 5
Mean	18. 6	99. 9	102. 4	107. 0

decrease in the rate of virus liberation, a finding encountered with all cultures which received this serum. When other serum was substituted, there was an increase in virus release.

After about 80 days, the rate had lessened so much that essentially no virus was released either by the culture in text-figure 3A or by that in text-figure 3B. On the 130th day, there occurred a marked renewal of liberation by culture 1 and at a rate essentially identical with that observed earlier. Between the 125th and the 185th days, an average of 3.7×10^9 particles were liberated per ml. per day in comparison with the value 3.8×10^9 during the period between the 18th and 52nd days. The 2nd culture did not show this activity. The single culture of series 2B, text-figure 3B, also resumed a high rate of liberation. In this case the rate between the 157th and 181st days was 3.3×10^9 particles per ml. per day as compared with 5.2×10^9 per ml. per day between the 6th and 46th days.

4. Variation in chicken serum.—Much variation was observed in the effects on virus release of chicken serum obtained in different lots. Tests made on 12 different batches of serum in 2 experiments yielded the results summarized in table 3. Experiment 1657 consisted of 5 pairs of cultures of cells from a single bird. The culture medium contained 0.5

TABLE 2.—*Rate of virus release in tissue culture during the first 48-hour culture period*

Percent serum	Virus particles/hr./ml. ($\times 10^6$)	Cells/ml. at 0 time ($\times 10^5$)	Virus/cell/hr.
Experiment 5156a			
5	9. 2	2. 61	35. 3
5	10. 0	2. 61	38. 3
5	12. 0	2. 61	45. 9
5	10. 1	2. 61	38. 7
5	10. 4	2. 61	39. 8
5	10. 8	2. 61	41. 4
Experiment 5256			
5	15. 1	6. 00	25. 2
5	10. 3	6. 00	17. 2
5	10. 6	6. 00	17. 7
5	11. 6	6. 00	19. 3
5	9. 6	6. 00	16. 0
5	14. 8	6. 00	24. 7
Experiment 5556			
20	14. 3	6. 67	21. 4
20	16. 1	6. 67	24. 1
20	15. 3	6. 67	22. 9
20	17. 3	6. 67	25. 9
20	15. 5	6. 67	23. 2
20	15. 3	6. 67	23. 0

TABLE 3.—*Variation in the influence of chicken serum of different batches on the release of the virus in cultures of myeloblasts*

Serum	Accumulative net virus particle production/ml.	
	Number ($\times 10^6$)	Percent of control
Experiment 1657*		
556 & 656 (Control)	212	
356	239	113
756	196	92
856	47	22
956	110	52
Experiment 2357†		
356 & 756 (Control)	38. 7	
456	31. 8	82
157	14. 2	37
257	16. 4	42
357A	15. 1	39
357B	11. 6	30
357C	12. 5	31
357D	14. 6	38

*Total culture period 5 days.
†Total culture period 6 days.

percent glucose and 20 percent serum of the lot indicated. A pool of serum from lots 556 and 656, known to support virus release at a high level, was used as control. The results showed that 3 of the batches, the control and lots 356 and 756, exerted similar effects. A difference was seen with lot 856, which was the serum associated with decline of virus liberation in the long-term cultures of text-figures 2 and 3.

Experiment 2357 was like 1657 but with cells from a different chicken and a pool of serum from lots 356 and 756 in the control cultures. Although the highest level of virus release was lower than that in the preceding experiment, the yield was highest with the control serum which, as already seen, consisted of 2 of the best lots in experiment 1657.

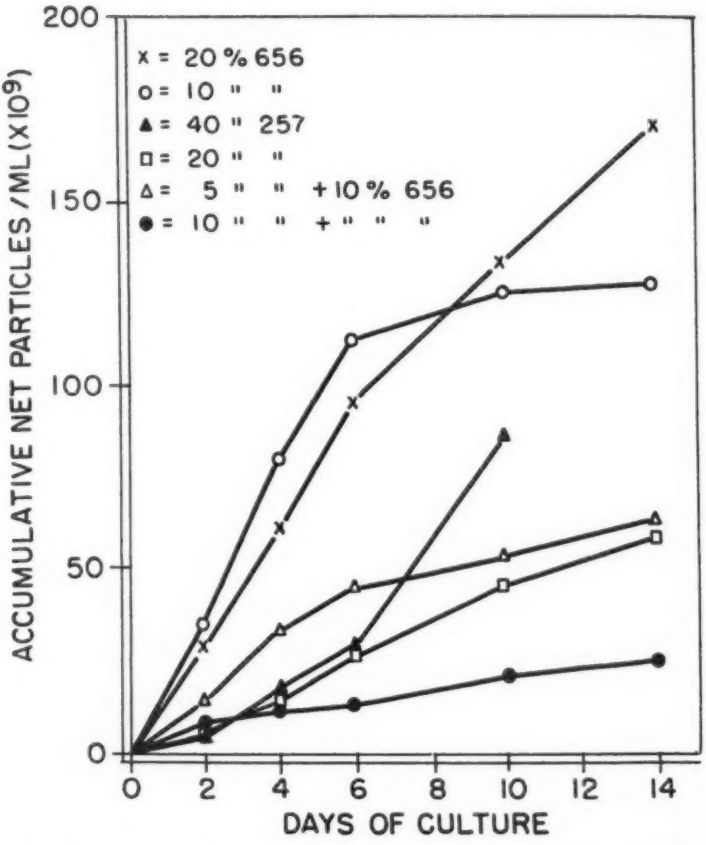
In these experiments the 7 lots of serum were obtained at different times from birds 5 to 6 months of age, but the chickens were not from the same hatch. Birds providing lots 157, 257, and 357, however, were from the same hatch. Lot 357 was separated into 6 different pools, each from 3 to 4 birds, and the 4 tested (expt. 2357) gave uniform results.

From the experience thus far it would appear that the adverse influence, that of lot 856 for example, was due to an inhibiting effect rather than to a lack of essential nutrient materials. Evidence supporting this possibility is seen in the results of the experiments summarized in text-figure 4. Six pairs of duplicate flasks were set up with cells in medium containing 0.5 percent glucose and 10 to 40 percent normal chicken serum in various proportions of two lots; namely, 656 and 257 (*see* table 3).

The largest total amount of virus was released in cultures containing 10 and 20 percent chicken serum of lot 656. The addition of either 5 percent or 10 percent of lot 257 to 10 percent of lot 656 inhibited virus liberation. This would appear to rule out a simple deficiency in the serum of lot 257, and suggests that the low rate of virus liberation was due to an inhibitory effect. That this effect was not complete was demonstrated by the results of using 40 percent serum of lot 257.

5. Other chicken serums and serums of different species.—In addition to normal serum from birds of line 15, several specimens of chicken serum obtained from an outside source (Microbiological Associates, Bethesda, Md.) were tested. Studies were made, also, with calf, ox, and horse serums, all in 20 percent concentration. In a typical experiment duplicate flasks were set up for each specimen of serum. It is seen in text-figure 5 that the serum of line 15 birds supported virus release much better than chicken serum obtained from other sources. The yield of virus was small in the media containing serums from the calf, ox, or horse. The addition of chicken serum to that of the other species did little to enhance the output of the agent.

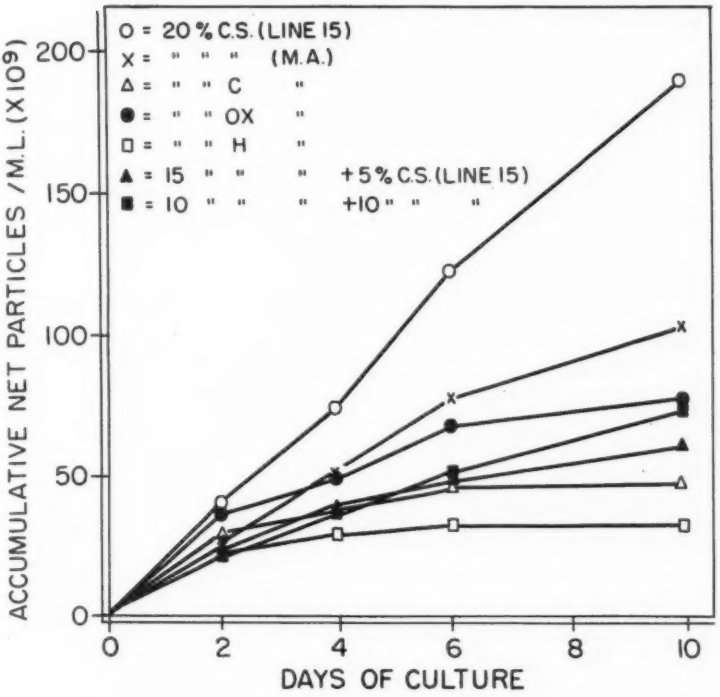
6. Cell concentration and virus release.—Cell concentrations suitable for the practical aspects of tissue culture under the conditions described were approximately 2.5 to 5.0 $\times$ 10^7 cells per ml. of fluid. With concentrations in this range, the cultures could be carried satisfactorily without change of the medium for at least 48 hours. When the number of cells was less, the output of virus per cell was increased, but the concentration



TEXT-FIGURE 4.—Inhibition of virus liberation by normal chicken serum 257 mixed in varying proportions with serum 656 which supported liberation at high levels (*see* table 3).

in the culture medium diminished to levels approaching the limits of the sensitivity of the method of enzyme measurement; namely, 915 million particles per ml. of fluid.

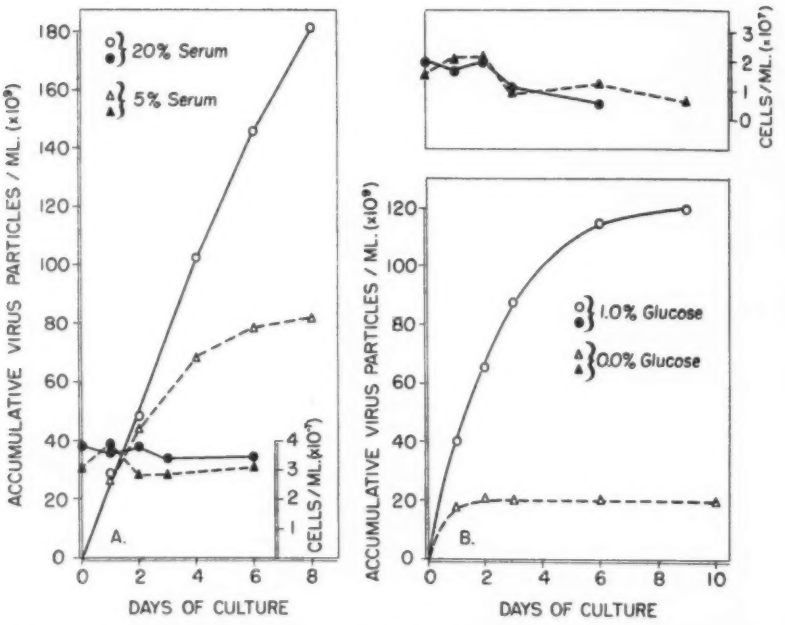
The effects of serum and glucose concentration on the number of cells initially introduced into the cultures in relation to the amount of virus released are summarized in text-figures 6A and 6B. Cultures were set up in quadruplicate. In one pair, text-figure 6A, the chicken serum content was 20 percent and in the other pair 5 percent. Despite the difference in the rates of virus release, text-figure 6A, there was essentially no change in the numbers of cells. A greater difference was observed, text-figure 6B, in an analogous study with a pair of cultures (5 percent chicken serum) containing no added glucose and another pair with glucose introduced in 1.0 percent concentration. The cultures without added glucose



TEXT-FIGURE 5.—Comparison of the effects of different chicken serums and of serums from other species on the rate and level of virus liberation. (M. A. = chicken serum from birds other than those of line 15; C = calf serum; Ox = ox serum; and H = horse serum. All serums except those from line 15 chickens were obtained from Microbiological Associates, Bethesda, Md.)

showed no increase in the agent after the first 2 days, but there was no significant difference between the number of cells in these flasks and that in the cultures containing 1.0 percent glucose.

7. Source of liberated virus.—In this system in which virus was associated with the cells before culture was instituted, direct demonstration of multiplication was not obtained by the studies thus far made. Nevertheless, no evidence could be found that virus in significant quantities existed in or was adsorbed on the cells before culture was begun. This could be investigated by measurements of the infectivity of extracts of myeloblasts obtained from the circulating blood. Additional information could be obtained by study of the relationships of infectivity to the adenosinetriphosphatase activity known to be associated with the virus and that derived by extraction of the cells. In such experiments it was observed, first, that the amount of virus extractable from the cells was negligible in comparison with the quantities released in culture. Furthermore, there was no relationship between infectivity and extractable



TEXT-FIGURE 6.—Lack of relation of virus release to cell number in cultures of different serum (A) and glucose (B) concentrations. The cell counts at the intervals noted are indicated in both graphs by the closed symbols.

enzyme nor was the quantity of cellular enzyme sufficient to account for the levels of virus released even in brief periods of culture. The findings may be illustrated by the data of the following experiments.

In one series of studies, measurements were made of the infectivity and enzyme activity of extracts of myeloblasts from the circulating blood. The plasma and the cells of two birds (P 740 and P 741) were separated and the cells washed 3 times in Gey's salt solution. 2.98 ml. of plasma from bird P 740 was diluted with 12.02 ml. of the saline, and 2.54 ml. of plasma from bird P 741 was diluted with 12.46 ml. of saline. Each diluted plasma was divided into 3 lots of 5.0 ml. each, and one aliquot from each bird was diluted to 10 ml. with Gey's salt solution and stored under CO_2 ice. A packed volume of 1.5 ml. of myeloblasts from bird P 740 was added to the second 5.0 ml. lot of diluted P 740 plasma, and 0.8 ml. of cells from bird P 741 was added to the second 5.0 ml. of diluted P 741 plasma. These cell suspensions were frozen and thawed 5 times in a CO_2-alcohol freezing mixture along with the third 5.0 ml. aliquot of diluted plasma from each bird. Not all of the cells were broken by this treatment, as microscopic examination showed. The cells and cell debris were sedimented, and the supernatant fluid was pipetted off. The sediment was washed twice with small volumes of salt solution, and the washings were added to the respective original supernatant fluids. The diluted plasma and cell contents

plus the washings were then diluted to 10 ml. and the diluted plasmas, which had been frozen and thawed 5 times (Plasma F and T), were also diluted to 10 ml. Infectivity and enzyme assays were made on the 3 samples from each bird with the results summarized in table 4.

TABLE 4.—*Relation of enzyme activity to infectivity in blood plasma and extracts of myeloblasts from the diseased birds*

Test material	Volume of inoculum (λ)	Chicks inoculated*	Chicks positive	Median log latent period (*days*)	Infectivity (*percent*)†	Enzyme activity (*units/ml.*)
Experiment 3457 (bird P 740)						
Plasma	1.0	30	23	1.188		42.4
	0.1	28	16	1.277		
Plasma (F and T)‡	1.0	29	22	1.172	120	39.5
	0.1	25	14	1.288	67	
Plasma+cells (F and T)‡	1.0	29	26	1.159	155	435
Experiment 3457 (bird P 741)						
Plasma	1.0	27	16	1.273		26.5
	0.1	25	6	1.342		
Plasma (F and T)‡	1.0	26	11	1.235	152	25.1
Plasma+cells (F and T)‡	1.0	27	15	1.256	96	222

*See reference (*20*).
†In relation to the infectivity of the plasma as 100 percent.
‡F and T signifies frozen and thawed.

The results of the 2 experiments were essentially identical. It was apparent that the treatment of freezing and thawing did not damage the infectious attributes of the virus as can be seen by the levels of infectivity of the treated plasmas which were actually higher, but not significantly so, than those of the untreated plasma. The notable feature of the experiments was the observation that the infectivity, and, in consequence, the virus content of the preparation containing the cell extract was not significantly higher than that of either the control or the treated plasma.

Similarly, freezing and thawing did not affect the measurable enzyme activity of the plasma. Treatment of the preparation containing cells, however, resulted in the release of enzyme tenfold that of the plasma, which yielded unequivocal evidence that extraction was effected. Nevertheless, as already indicated, there was no relationship between the enzyme thus released and infectious virus. It is notable that, in culture, the number of cells used in experiment 3457 would have been expected to have released 600 enzyme units of virus (employing the average value 31 particles per cell per hour) within a single 48-hour period. It is obvious that virus in

such amount was not associated with the extracted adenosinetriphosphatase.

Further evidence that the virus released into the medium must have been produced by the cells during culture was obtained by measurement of the enzyme of the cells before and after culture and comparing the quantities of cellular enzyme with those appearing free in the fluid in association with virus. Data were obtained by 2 procedures.

With the first, myeloblasts were washed 3 times as usual, and cultures were set up with 20 percent serum and 0.5 percent glucose. After the culture intervals designated in table 5, samples were removed for cell counts and enzyme measurements on the culture fluid. The remaining suspension of cells in the culture medium was frozen and thawed 3 times in an alcohol-CO_2 freezing mixture, which resulted in the breakage of practically all the cells as determined by microscopic examination. Cellular residue was sedimented by centrifugation, and measurement was made on the total enzyme activity of the supernatant fluid. Cell enzyme activity was then calculated by difference from the value observed with the cell-free culture fluid.

In the alternate procedure, activity was measured directly on aliquots of the cells before and after culture. Cells separated from the culture medium by centrifugation were washed 3 times in balanced salt solution and resuspended in the saline in volumes equal to those of the culture fluids from which the cells were derived. Cell counts were then made, and the cells were broken by repeated freezing and thawing as before. In these determinations there were thus separate values for the virus in the medium and for enzyme in the extracts of broken cells. The data obtained with this procedure are designated in the table as "washed cells."

In these experiments it was revealed, first, that the total enzyme content of the cells did not diminish during culture periods of 24 or 48 hours. Indeed, the amount of extractable enzyme per cell was uniformly greater after a period of culture than before. In addition, it is seen that the amount of enzyme liberated into the medium as virus was equal to, or greater than, the enzyme of the cells at the time culture was begun. In 2 of the 48-hour preparations, the amount of enzyme measurable as virus was about fivefold that of the intracellular enzyme at the beginning of the culture periods. From these findings it is clear that adenosinetriphosphatase was elaborated by the cells during culture and that large proportions of it were liberated into the medium as myeloblastosis virus.

8. The inhibition of virus release by dinitrophenol.—Dinitrophenol inhibits the growth of cells and restricts protein synthesis at concentrations of 10^{-4} to 10^{-6} *M* in tissue-culture medium. The material has been shown, likewise, to inhibit virus multiplication (*23*, *24*).

Dinitrophenol in like concentrations inhibits liberation of myeloblastosis virus. In 2 experiments, about 2.5×10^7 cells per ml. were cultured in medium containing 5 percent serum and 0.1 percent glucose to which was added dinitrophenol in the concentrations listed in table 6. The net

TABLE 5.—*Adenosinetriphosphatase activity of myeloblasts before culture and after culture and of the virus liberated by the cells during culture*

Flask	Culture period (*days*)	Enzyme units/ml.		Cells/ml. ($\times 10^7$)	Enzyme activity/cell ($\times 10^{-7}$)
		Culture medium	Cell extract		
Experiment 1					
	0	0	29. 2	2. 95	9. 9
1	1	35. 7	44. 9	3. 60	12. 5
11	1	29. 6	46. 2	3. 28	14. 1
2	2	58. 2	55. 5	3. 74	14. 8
10 (W C)*	2	70. 4	39. 8	3. 96	10. 1
Experiment 2					
	0	0	14. 9	2. 88	5. 2
1	1	35. 0	43. 1	3. 78	11. 3
2	2	77. 6	71. 0	3. 64	19. 5
11 (W C)	2	67. 8	57. 9	3. 33	17. 4
Experiment 3					
	0	0	50. 4	5. 12	9. 9
1	2	45. 6	94. 6	6. 84	13. 8
Experiment 4†					
	0	0	15. 7	5. 30	3. 0
1 (W C)	2	52. 1	20. 0	4. 59	4. 4
Experiment 5					
	0	0	54. 6	7. 42	7. 4
1 (W C)	2	106. 4	110. 1	7. 38	14. 9
Experiment 6					
	0	0	27. 1	5. 24	5. 2
1 (W C)	2	73. 9	73. 1	5. 54	13. 2

*W C=washed cells (*see* text).
†The cells of this experiment were obtained from a bird of low concentration of myeloblasts in the blood.

virus liberation by the cells during the first 24 hours is expressed in terms of enzyme units. In a series of control experiments without cells, the activity of virus incubated under otherwise similar conditions both in the presence of dinitrophenol and in the absence of the material decreased from 18 to 20 percent during the 24-hour period. Therefore, it may be assumed that the presence of the inhibitor, *per se*, did not affect the outcome of the assay. In table 6 it is seen that progressive increase in the concentration of dinitrophenol in the cultures resulted in decrease in virus liberation.

TABLE 6.—*Inhibition of virus liberation by dinitrophenol in 24-hour cultures*

Dinitrophenol ($\times 10^{-4}$ *M*)	Enzyme units liberated per ml. culture fluid		Inhibition (*percent*)
	Individual	Mean	
Experiment 1			
0	5. 7		
0	6. 1	5. 9	
5	4. 0		
5	4. 0	4. 0	32
25	1. 6		
25	2. 1	1. 85	69
50	0. 6		
50	2. 3	1. 45	75
100	1. 0		
100	0. 6	0. 8	86
Experiment 2			
0	19. 2		
0	20. 6	19. 9	
5	18. 5		
5	13. 3		
5	13. 7		
5	12. 3		
5	14. 8		
5	13. 8	14. 4	28
25	8. 7		
25	8. 2		
25	10. 1		
25	11. 7		
25	15. 0	10. 7	46
50	9. 2		
50	11. 2	10. 2	49
100	0		
100	0	0	100

9. Infectivity of virus liberated into the culture fluid.—Thus far, the principal criterion of the course of virus release by the myeloblasts in tissue culture has been that of enzyme activity standardized against direct particle count. In some of the experiments already described, studies were made to relate infectivity of the culture fluids to enzyme activity and particle number. The character of the experiments and the data accumulated are shown in table 7.

All of the materials studied, with the exceptions indicated later, were passed through Selas 02 or 015 filters (*7*). All had been frozen in an alcohol and CO_2-ice bath and stored in rubber-capped vials under cracked CO_2 ice. Myeloblastosis virus in 2 different pools of plasma, C and D, was employed as control to establish the level of host response. Another

TABLE 7.—*Infectivity of virus in fluids of cultures of myeloblasts and relationship of number of virus particles estimated by infectivity with results of estimates obtained by enzyme activity and direct particle count*

Material	Total culture period (*days*)	Infectivity measurements				Particles per inoculum ($\times 10^9$)		
		Volume inoculated (λ)	Chicks inoculated*	Chicks positive	Median log latent period (*days*)	Infectivity	Enzyme activity	Direct particle count
Experiment 5156b								
Control C		10	28	23	1. 158		7. 7	9. 9
"		1	25	15	1. 248			
Control C (incubated)	2	10	30	0			4. 8	9. 5
"		1	30	0				
"0" fluid	0	100	30	0			no reaction	0. 8
Pool 1	2	100	29	12	1. 301	0. 33	3. 3	9. 8
"	2	10	27	10	1. 384	0. 054		
Pool 2	2	100	30	9	1. 356	0. 10	0. 8	0. 2
"	2	10	30	4	>1. 4	<0. 04		
Experiment 5256								
Control C		10	29	22	1. 128		8. 3	9. 5
"		1	26	17	1. 218			
Control C (incubated)	2	10	30	0			3. 4	12. 0
"0" fluid	0	100	30	0			no reaction	
Pool 1	2	100	26	19	1. 187	2. 1	2. 3	2. 4
"	2	10	26	21	1. 274	0. 33		
Pool 2	2	100	30	15	1. 218	1. 1	3. 7	2. 2
"	2	10	27	17	1. 308	0. 16		

TABLE 7.—*Infectivity of virus in fluids of cultures of myeloblasts and relationship of number of virus particles estimated by infectivity with results of estimates obtained by enzyme activity and direct particle count*

Material	Total culture period (*days*)	Infectivity measurements				Particles per inoculum ($\times 10^9$)		
		Volume inoculated (λ)	Chicks inoculated*	Chicks positive	Median log latent period (*days*)	Infectivity	Enzyme activity	Direct particle count
Experiment 5556								
Control C		10	28	18	1. 187			10
"		1	24	9	1. 302			
Control C (incubated)	2	10	28	0				
"0" fluid	0	100	28	4	>1. 4	<0. 08	no reaction	0. 1
Pool 1A	2	100	27	17	1. 128	39. 5	4. 6	12. 4
"	2	10	28	12	1. 252	2. 7		
Pool 2A	2	100	26	12	1. 211	6. 3	3. 0	5. 9
"	2	10	30	10	1. 288	1. 2		
Experiment 5556								
Control C		10	29	18	1. 178			10
"		1	28	12	1. 289			
Pool 1B	2	100	30	20	1. 178	8. 0	3. 4	9. 3
"	2	10	29	16	1. 282	0. 8		
Pool 2B	2	100	27	13	1. 263	1. 2		
"	2	10	26	12	1. 355	0. 15		
"	6	100	29	17	1. 227	2. 7	0. 8	2. 2
"	6	10	27	7	1. 352	0. 17		

Experiment 5556								
Control D		10	27	16	1. 147		6. 9	
"		1	30	15	1. 31			
Pool 2A	24	100	30	6	>1. 4	<0. 04	1. 0	
"	24	10	27	3	>1. 4	<0. 04		
Pool 2B	24	100	28	5	>1. 4	<0. 04	1. 3	
"	24	10	28	5	>1. 4	<0. 04		
Pool 2A	26	100	27	6	>1. 4	<0. 04	0. 5	
Pool 2B	26	100	27	4	>1. 4	<0. 04	1. 1	
Experiment 5556								
Control C		10	29	21	1. 143			10
"		1	24	7	1. 302			
Pool 2A	175	100	25	5	>1. 4	<0. 06	2. 5	
"	181	100	26	7	>1. 4	<0. 06	1. 6	
Experiment 5456								
Culture 1	179	0	28	10	>1. 4	<0. 06	2. 5	
"	185	100	24	14	>1. 4	<0. 06	1. 8	

*See (20) for method of calculation.

control system consisted of plasma of Pool C or D diluted with culture medium and incubated on the shaker at 37° C. In each of the experiments, a vial of 1 or the other pool was thawed and prepared for injection just before the inoculations were made. The "0" fluid employed in each case was a sample of the culture medium taken immediately after the washed cells had been added. Calculation of particle number from the infectivity data was made on the basis of the potency ratio between the activity of the control and that of the test specimens determined from median log latent-period relations (*20*) and particle number of the control as estimated by direct particle count.

Experiment 5156b involved 4 cultures containing 5.9×10^7 cells per ml. in a medium of 5 percent serum and 0.5 percent glucose. The cells were all from the same bird (L 88). The fluids of the 4 cultures were combined in pairs to make 2 pools, 1 and 2. The values cited in table 7 were obtained by assay of the separate pools.

Experiment 5256 was decribed in section 1. Pools 1 and 2 of table 7 consisted of the fluids giving the results of text-figures 1B and 1D, respectively.

Experiment 5556 (*see* table 2) comprised 12 cultures containing 6.67×10^7 cells per ml. in 20 percent serum and 0.5 percent glucose. The cultures were carried as 4 separate groups of 3 flasks each. With 2 of these groups, designated as Pools 1A and 1B of table 7, fluid changes were made every 4 hours for a 2-day period. At the first change, the cells were sedimented, and the fluid was replaced with fresh medium. The culture fluids taken off were pooled in the 2 groups; the virus was sedimented by spinning at 28,800 × g for 1 hour in a refrigerated centrifuge; and the supernatant fluid was pipetted off under aseptic conditions. The pellets containing the virus were resuspended in less than 1.0 ml. of fresh culture medium, sealed in glass and stored under CO_2 ice. The supernatant fluid was returned to the cultures at the next fluid change. This routine was continued for the 12 changes. In this way the virus produced by the cells was removed at 4-hour intervals, yet the cells were maintained in a "conditioned" medium. At the end of 48 hours, the resuspended pellets were combined in the respective pools and diluted to 9 ml. with fresh medium for the various assays.

The other 6 cultures were likewise carried in 2 groups of 3 flasks each, Pools 2A and 2B of table 7. Fluid changes were made at 48-hour intervals until the 8th day and thereafter every 4 days. At each change the fluids were pooled in the usual way, and the infectivity and other measurements were made on the fluids collected at the designated intervals.

The infectivity measurements on Pool 2A (expt. 5556) at 175 and 181 days and on culture 1, experiment 5456, section 3 (text-fig. 3A), at 179 and 185 days were made on fluids which were not filtered but were spun once at 150 × g for 10 minutes and 3 times thereafter at 2,000 × g for 20 minutes.

The results cited in table 7 reveal, first, a confirmation of the general finding in the short-term cultures of a close relation between the number of

virus particles found by enzyme activity and that determined by direct count. Differences were relatively wide in some instances, though no greater at the extremes than threefold. With respect to measurement by assay of infectivity, the biologic activity of the agent in one experiment, 5156b, was approximately tenfold less than that expected on the basis of particle count by the other methods. In experiment 5256, however, the relationship between the 3 values was excellent. Another extreme was seen in experiment 5556 in the infectivity values obtained with Pool 1A which were higher than expected. Nevertheless, the remainder of the data showed relationships not greatly different from those evident in experiment 5256. Virus exhibiting infectious activity was found throughout the periods of 181 and 185 days of culture.

10. Morphology of the tissue-culture virus.—Within the limits of the information provided by pictures of the particles shadowed with metal, the virus released in culture did not differ from that seen in the plasma of diseased birds (*22*). A picture of the agent from a 48-hour culture (expt. 5156b, Pool 1, table 7) is shown in figure 1 and another at higher magnification comprises the micrograph of figure 2. It is notable, here, that little material other than the characteristic particles is to be seen.

With decline in the rate of virus liberation after 2 to 12 days, the concentration of particles in the fluid of a given culture interval was much less, and the pictures showed correspondingly fewer particles. This is evident in figure 3 which reveals the results obtained with the fluid of a 20-day culture (2.0×10^9 particles estimated by enzyme activity and 2.2×10^9 by count). The images were still of the same appearance as that of figure 1 but fewer in number.

The fluids employed for the studies just described had been filtered before examination. Pictures were obtained with the unfiltered fluid of a 179-day culture (culture 1, expt. 5456 of table 7, and text-fig. 3A). An electron micrograph of this fluid, figure 4, revealed few particles of characteristic appearance and much amorphous material. Although some of the particles were obviously like those seen, for example, in figures 1 and 3, counts could not be made with assurance. Nevertheless, the micrograph supplied definite physical evidence of the presence of the virus demonstrable by enzyme activity and infectivity.

Discussion

Experience with the primitive cells of avian myeloblastosis in tissue culture has revealed a system of unique potentialities for investigation on the interrelationship of host cells and virus responsible for the induction and maintenance of the state of malignancy. Although certain restrictions to fully comprehensive work were imposed by natural biologic principles inherent in the system, the results already encountered are sufficient to indicate advantages for study which thus far are remote for any other tumor virus and, indeed, are unparalleled by any other filterable agent. Many difficulties attend work with tumor viruses. Foremost among the impediments is the lack of the means for accurate and rapid bioassay

requisite for large-scale quantitative studies. Actually, except for the agent of rabbit papillomatosis (*25*, *26*), unequivocal identification of the agents responsible for the various virus tumors has not been attained. It has been only recently that good evidence has been obtained of the probable morphologic characteristics of the most important tumor viruses, those of the Rous and other chicken sarcomas (*27*, *28*); the mouse mammary carcinoma (*29*, *30*); and the agents of lymphocytic leukemia (*31*) and associated conditions of mice. Such is not the case with myeloblastosis. The agent has been identified (*22*) in its physical state outside the cells, and many of its physical, chemical, and biologic characteristics have been determined. Of major significance for the present work is the adenosinetriphosphatase activity quantitatively proportional to virus-particle number which gives access to procedures for accurate measurement of virus amount. Related advantages are the high stability of the enzyme and the lack of reaction of specific immune bodies with the enzyme (*22*, *32*, *33*). In addition is the unusual condition of high concentration of virus reached both in the plasma of diseased birds (*3*, *19*, *21*) and, most fortunately as well, in the tissue-culture fluids. The concentration is such that not only enzyme measurement is applicable, but the particles are in sufficient numbers to permit direct counts in the electron microscope for the frequent correlations needed for quantitative interpretations. Finally, there are available titration procedures (*20*) with hosts in suitable numbers and of appropriate genetic constitution (*12*, *13*) for the correlation of the chemical and physical data with specific biologic activity.

It would be well to consider further the characteristics of the enzyme reaction as an index of virus quantity. The status of both the quantitative and qualitative aspects of this question has already received much attention (*3*, *19*, *21*) in relation to virus occurring in the plasmas of diseased birds. An initial question in the present work was the relation of particle number to enzyme activity in the culture fluids. The excellent agreement of data obtained with the unrelated procedures is well illustrated in text-figure 1. Under controlled conditions involving the use of a stable standard of reference, the data could be corrected to absolute numbers for comparisons from one experiment to another over a long period of time. The only serious defect in the procedure is lack of sensitivity, one shared by both particle counting and assay of infectivity. The unit of enzyme activity, chosen arbitrarily as a matter of convenience, was that represented by a reaction period of 1,000 seconds which corresponded to a concentration of 915 million particles per ml. of test fluid. This was approximately the lower limit of particle concentration likewise necessary for particle counting. With respect to infectivity, something like 24 million particles are needed (per 0.1 ml. inoculum) to induce disease in 50 percent of the chicks inoculated (*34*). Numbers of particles below these levels are simply beyond the limits of measurement without recourse to concentration procedures too time-consuming for routine investigation.

The tissue-culture studies on myeloblasts differed in one very significant respect from those involving most other viruses; namely, the absence of the phenomenon of induced infection of the host cell. The myeloblasts already carried their parasite, and the observations with long-term cultures suggested that the state of parasitism is continuous and mutually compatible with the respective processes of cell metabolism for indefinite periods. There is certainly no obvious cytopathogenic effect, and the cells continue to flourish and undergo mitosis despite the regular liberation of large numbers of virus particles. It is significant, however, that the cells both in the host and in the cultures remained myeloblasts and showed no signs of differentiation into the mature forms of myelocytes or change into other types of cells.

Because of the nature of the system, nothing was learned about possible "cycles" of virus multiplication or liberation. The data indicated only a steady liberation of particles and at a rate which seemed surprisingly large. It could be shown, in some groups of cultures from the cells of a single bird, that as many as 40 particles per cell per hour reached the culture fluid, a total of almost 2,000 particles per cell in the initial 48-hour period. Occasionally the rate was essentially linear for 8 to 12 days, and the total amount of virus liberated per cell was about 4,500 or more particles. Over longer periods, with the much lower rates, the total amount of the agent produced by the cells initially seeded in 1 ml. of medium was at least 400 billion particles in about 50 days, an output of about 8 billion per day per ml. of fluid (40 billion per total culture). In some of the long-term cultures initially seeded with 2.5 to 5 $\times$ 10^7 cells per ml., the total output per culture was about 3 trillion particles in 6 months. It is notable that these computations were made on the basis of data of considerable precision as cited from the various tables and charts shown above.

Thus far, all of the cultures carried for long periods have shown a decreasing rate of virus liberation after about 6 weeks, a behavior not necessarily characteristic of the system. Unfortunately, the phenomenon was associated in all instances with the use of serum which adversely affected all cultures receiving it. Although slight recovery was observed by transfer to other serum, virus release soon ceased or was carried on at a very low level. In 2 experiments after a prolonged period of quiescence, there occurred a resumption of virus formation at rates essentially identical with those seen in the first 6 weeks. The reason remains obscure, but the phenomenon suggests the possibility that conditions may be found which will be optimal for continued production at high rates.

Unequivocal evidence of multiplication of the virus in the myeloblasts during culture has not been available from results of the experiments thus far made. It is conceivable that the agent subsequently released into the medium was adsorbed on the cell surface or that it might have existed as mature virus within the cell before culture was instituted. There was much evidence, however, to suggest that the process of liberation was associated with concurrent virus synthesis. The agent in amounts of

the order of those released was not demonstrated in extracts of the myeloblasts obtained directly from the blood. This was shown by the low order of virus infectivity in the extracts of the cells as shown in table 4. That technical difficulties interfere with separation of virus from cellular material is well recognized (*35*); nevertheless, it seems unlikely that virus in the quantities released into the culture fluid could have escaped detection in the extracts.

Further evidence that virus formation was a continuous process was found in the observation that the level of adenosinetriphosphatase content of the myeloblasts did not decline during culture, while, at the same time, the output of the enzyme as virus exceeded manyfold the enzyme content of the cell at the beginning of culture. The cell population was not stable but was maintained as a balance between cell degeneration and multiplication throughout the longest periods of culture as shown by the presence of mitotic figures. Virus liberation was affected by factors, serum and glucose, influencing the nutritional status but not the number of cells in limited periods of culture. The agent was liberated steadily for long intervals suggesting a process of progressive and continuous virus synthesis. The agent must have multiplied at one time or another in the myeloblasts. It would be difficult to understand why the cells should have supported synthesis until the moment tissue culture was begun and then abruptly ceased to function in this process. Finally, dinitrophenol has been found to interfere with the multiplication of other viruses, and the material likewise suppressed liberation of the myeloblastosis virus from myeloblasts in culture.

A final point of much significance in the present results is the small contribution of infectivity measurements to the understanding of the happenings in tissue culture. While it could be shown by other means that virus was being liberated at a high rate, titration was useful only for *detection and proof of presence*, but not for *quantitative interpretation* except under the conditions of high virus concentration in the earliest periods of culture. Even here, interpretations were possible only on the basis of correlations established by particle count and enzyme measurement. It is clear that it would have been impossible to have gained significant information about the amount of virus released by titration alone. The conditions in myeloblastosis are somewhat extreme but not greatly so in comparison with the agents of papillomatosis and of erythroblastosis. As mentioned before a few studies have indicated an ID50 of 24 million particles of myeloblastosis (*34*). Repeated studies have revealed the need for about 80 million particles of papilloma virus (*34*) to produce the 50 percent point of infectivity. Erythroblastosis virus is much more "virulent" requiring (*36*) approximately 100- to 1,000-fold less of the agent for the ID50. It is known that different agents vary greatly with respect to host response. At the extreme, opposite the tumor viruses mentioned, is the bacteriophage with which, indeed, host response is almost perfectly related (*37*) to the individual virus particle. In-between lie great differences with other agents. The

results with myeloblastosis serve to emphasize the need for caution in quantitative interpretations of virus multiplication in tissue culture based only on infectivity measurements.

Summary

A study has been made of the myeloblasts of avian myeloblastosis in tissue culture. The cells can be maintained for periods of at least 6 months during which the agent of the disease was liberated continuously into the culture fluid. Evidence that the virus multiplied in the cells during culture was discussed. Measurements of virus output were made by determinations of enzyme activity of the agent to dephosphorylate adenosine triphosphate and by direct enumeration of the particles in the electron microscope. Virus liberation was greatly dependent on chicken serum in 20 percent or greater concentration in the medium and on the presence of added glucose. The process was inhibited by dinitrophenol.

The rate of virus liberation was greatest during the earlier periods of culture from 2 to 12 days, during which the average output was about 31 particles per cell per hour (1,500 particles per cell in 48 hours). Later the process slowed somewhat, but in some cases was resumed at a high level after periods of quiescence. In some 5 ml. cultures initially seeded with 2.5 to 5 $\times$ 10^7 cells per ml., the total virus output was about 3 trillion particles in 6 months. Multiplication of the myeloblasts themselves was a continuous process as indicated by mitotic figures, but the increase was not sufficient to permit division of the cultures.

Electron micrographs revealed no difference in the morphology of the culture virus from that found in the plasma of diseased birds.

It is evident that cultures of the myeloblasts provide a relatively stable and controllable system for extensive study of the conditions conducive to the liberation from the host cell of an agent responsible for the direct induction of the malignant state.

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PLATE 37

FIGURE 1.—Electron micrograph of myeloblastosis virus from the filtered fluid of a 48-hour culture of myeloblasts. × 14,500

FIGURE 2.—An area of figure 1 at a higher magnification. × 39,800

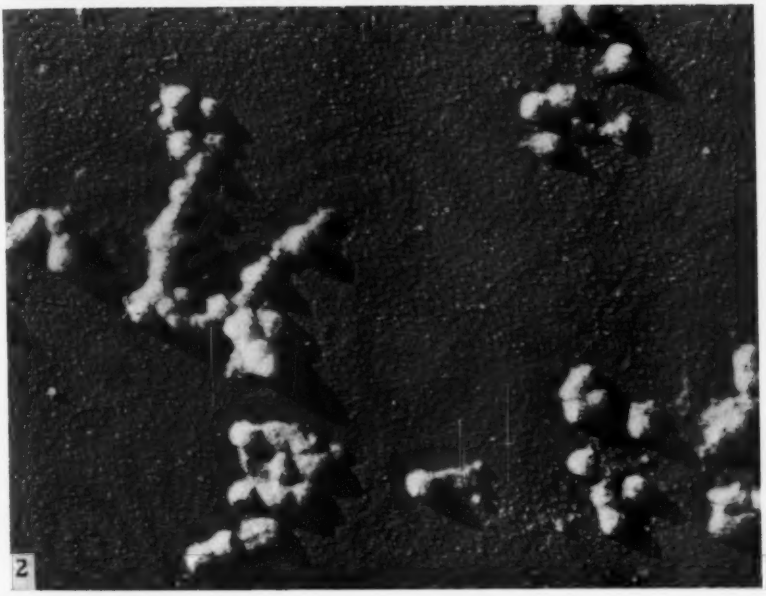

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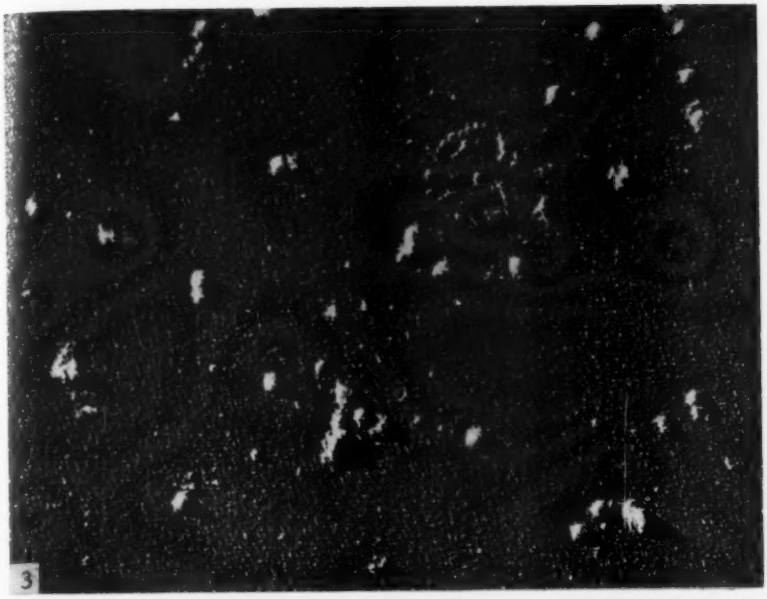
PLATE 38

FIGURE 3.—Electron micrograph of myeloblastosis virus from a filtered fluid of a 20-day culture of myeloblasts. × 26,900

FIGURE 4.—Electron micrograph of myeloblastosis virus from the unfiltered fluid of a 179-day culture of myeloblasts. × 26,900



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Comparative Chromosomal Studies on Mammalian Cells in Culture. I. The HeLa Strain and Its Mutant Clonal Derivatives [1, 2, 3]

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The development by Puck and his associates (*1*, *2*) of efficient methods for obtaining clonal lines of mammalian cells opens many possibilities for cytogenetical studies with such materials. These investigators have observed that the clones isolated from the human cervical carcinoma strain HeLa (*3*, *4*) exhibit much greater uniformity of behavior than does the parental strain, and that different clones exhibit differences in characteristics, such as plating efficiency in various media. They have further noted that these differences have persisted throughout more than 20 passages and have designated such strains as "mutants." [5]

The nature of the inferred mutational changes responsible for these new clonal derivatives is a matter of considerable cytogenetical interest. Since the principal cell strain used in these studies, HeLa, has been reported (*5*) to be cytologically abnormal in that its cells have varying numbers of chromosomes with a peak frequency between 80 and 90, a cytological examination of its derivative mutant clones seemed especially desirable. It appeared possible that some or all of the clones might be cytologically distinguishable from the parental line on the basis of their chromosomal constitutions. Such information should aid in elucidating the mechanisms responsible for the origin of these mutant clones, as well as for their subsequent behavior.

Materials and Methods

The parental HeLa strain from which the clones were isolated was obtained from the George Washington Carver Foundation, Tuskegee

[1] Received for publication July 31, 1957.

[2] Supported in part under contract AT(30-1)-1908 with the Atomic Energy Commission.

[3] The clonal cell strains used in this study were furnished on a cooperative basis by Dr. Theodore T. Puck and his colleagues at the Department of Biophysics, University of Colorado Medical Center, Denver, who are conducting radiobiological investigations on somatic mammalian cells. The properties and genetic relationships of these mutants, a description of the radiation procedure employed, and details of the clonal isolates are appearing in a series of articles, some of which have already been published (*6–8*). The authors gratefully acknowledge the generosity of Dr. Puck in furnishing these clonal cell strains and his kindness in reviewing the manuscript.

[4] The authors wish to thank Dr. Arthur W. Pollister for permission to use his laboratory facilities and Dr. Ruth Kleinfeld for her cooperation in microspectrophotometric measurements. They also wish to acknowledge the technical assistance of Miss Jean McCauley.

[5] A new term "genome-mutant," which is defined as any alteration of genetic constitution in a somatic cell, has recently been introduced by Dr. Puck (*7*).

Institute, Alabama. The clonal isolates used in the present study were made available through the generous cooperation of Dr. Theodore Puck. S1 and S3 are two clonal mutant isolates exhibiting genetic differences in their responses to different media (*9*), while S3–R3, S3–RA1, and S3–RE1 are mutant clones derived from S3. These last 3 clones are part of a series picked from among radiation survivors (600–900 r) and have been found to have altered biochemical growth requirements (*10*). The morphology of HeLa and all these clonal derivatives is epithelium-like.

S1 and S3 were isolated as clones on April 11, 1955 and May 6, 1955, respectively, while the 3 derivatives from S3 were isolated on January 1, 1956. The number of passages can be computed on the following basis: 1 week = 1 passage = 2 generations (*i.e.*, a fourfold increase in progeny).

The procedures for growing mammalian cell cultures and for their cytological study have been described in a previous publication (*11*). Standard mass-culture practices were followed for these cell strains. Incubation temperatures were set at 35° C. for rapid proliferation and at 30° C. for maintenance. The medium used consisted of 90 percent Eagle's synthetic medium (*12*) in which 10^{-2} mM per l. of inositol was included (*13*) and 10 percent human or horse serum. Cells were grown on the glass surfaces of ordinary 3-ounce prescription bottles or on removable 22 × 11 mm. coverslips in depression test tubes (Leighton tubes).

For the study of mitotic activities, a 1 ml. cell suspension with approximately 100,000 cells was seeded into each tube. The coverslips were removed from culture tubes, fixed for at least 24 hours in 10 percent neutral formalin, and stained by the Feulgen procedure, with or without fast green counterstaining (for 1 minute in a 0.05% aqueous solution).

For deoxyribonucleic acid (DNA) measurements, coverslips of the strains to be compared, all in 3rd-day cultures, were put in a single porcelain coverslip tray and passed simultaneously through the Feulgen procedure in order to assure uniform staining. Microspectrophotometric measurements of the Feulgen-stained nuclei were made by means of the two-wavelength method (*14*, *15*).

For chromosomal studies, cells both on coverslips and in suspension can be used. In the latter case, cells were removed from walls of culture bottles by a brief incubation with trypsin or versene solutions, pretreated with hypotonic saline to spread the chromosomes, fixed and stained with acetocarmine, and thereafter squashed. For cells grown on coverslips, hypotonic treatment was performed inside the tube.

In order to accumulate metaphases for chromosome analysis, colchicine pretreatment was employed. A 10^{-4} gm. per l. colchicine solution, equal to one tenth the volume of the culture medium, was added to each culture vessel 8 to 12 hours before fixation. This made the colchicine concentration about 10^{-5} gm. per l. (or 25×10^{-9} M per l. for colchicine).

Chromosome counts were made from single, intact cells at metaphase or prometaphase, during which the chromosomes were widely spread. A sketch was made of each cell counted, in order to check the chromosome number as accurately as possible. However, in view of the high chromo-

some numbers in these cells, counting errors of 1 or 2 chromosomes per cell may occur. The idiogram analyses in figures 1 and 2 were drawn with a camera lucida. Each plate was first drawn at a magnification of 10 × 90. Thereafter, the chromosomes were numbered and each of them was moved into the center of the view field and outlined again at 15 × 90 magnification. The chromosomes have been arranged roughly according to size. Although it is impossible to identify individual chromosomes, those in the same size range should be comparable. Photomicrographs were taken with a Leitz Ortholux-Aristophot unit and magnifications are indicated under each figure.

Chromosome Number and Morphology

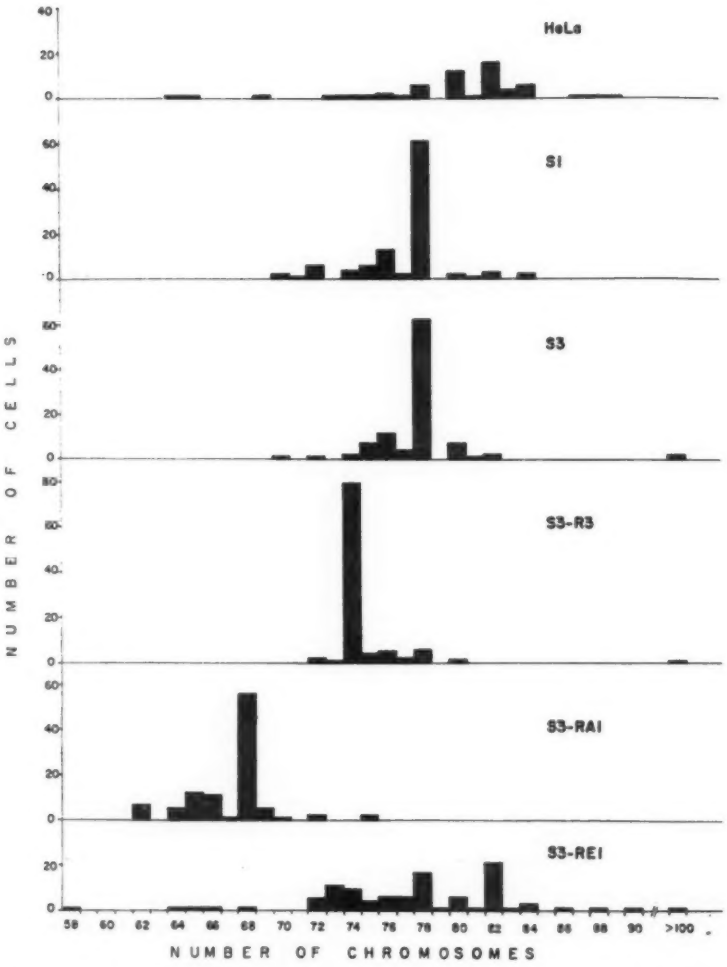
The results of chromosome counts on the HeLa strain (Carver Foundation culture) and on 5 clones derived from it are presented in table 1 and as histograms in text-figure 1. In addition, previous results of an examination of the HeLa strain (maintained at the University of Texas, Medical Branch, Galveston) by Hsu (*5*) are given for comparison.

It is evident that the chromosome number in cells of the HeLa strain varies widely on the basis of Hsu's observations, a conclusion with which our (more limited) observations are in substantial agreement. However, in the present counts there is a shift in the frequency distribution toward lower chromosome numbers.

Furthermore, it is also clear that 4 of the derived mutant clones are distinguishable from the HeLa strain on the basis of present counts. These 4 strains show a marked reduction in chromosome-number variation. All except S3-RE1 may be considered to exhibit a modal or stemline condition.

S1 and S3 have the same stemline number, 78. This does not necessarily mean, however, that chromosome-number variation can be eliminated as being responsible for the genetic differences distinguishing them. Since it still seemed possible that their chromosome complements might be different, an attempt was made to detect such differences.

Idiograms of one S3 cell and one S1 cell are shown in figures 1 and 2. The S3 cell has been pretreated with colchicine, which is responsible for the slight shortening of chromosome lengths and spreading of arms of chromatids. It appears that the chromosome complements of these 2 clones generally correspond. However, detailed comparisons have revealed that they differ in certain respects. Using the system of Tjio and Levan (*16*), chromosome arm indices of each individual chromosome of the 2 clones were calculated. The chromosomes are classified arbitrarily into 3 groups: M chromosomes (median-submedian centromeres), S chromosomes (subterminal centromeres) and T chromosomes (nearly terminal centromeres). Thus, the S3 cell consists of 31 M chromosomes, 43 S chromosomes, and 4 T chromosomes, while the S1 cell contains 30 M, 46 S, and 2 T chromosomes. Furthermore, all 4 nearly telocentric chromosomes in the S3 strain are medium-sized, while the 2 telocentric chromosomes in S1 are relatively small in size.



TEXT-FIGURE 1.—Distribution of chromosome numbers in HeLa and its 5 clones.

S3-R3 seems to be the most stable clone as far as the chromosome number is concerned, with 79 percent of the cells having 74 chromosomes. In S3-RA1, 68 is the stemline chromosome number, representing about 56 percent of the cells examined.

An interesting dicentric chromosome of spontaneous origin has arisen in S3-RA1 (fig. 3) during the course of this investigation. At this point, it should be mentioned that all the clones were obtained from Dr. Puck in mid-November, 1956, approximately 1 or 1½ years after they were isolated. Cytological studies were carried out in this laboratory during the 3- to 4-month period following shipment. There were no appreciable changes in the frequency distributions of chromosome numbers in all

TABLE 1.—*Frequency distribution of chromosome numbers in HeLa and its clonal derivatives*

Strain	No. of chromosomes																																No. of cells analyzed
	57 to 61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91 to 100	over 100	over 200	
HeLa..... (Hsu)															18									42						16	17	7	100
HeLa..... (Carver Found.)				1	1				1				1	1	1	2	1	6		12	1	16	4	6			1	1	1				57
S1.....										2	1	6		4	6	13	2	61		2	1	3		2									103
S3.....										1		1		2	7	11	4	62		7	1	2									2 (113) (150)		100
S3-R3.....												2	1	79	4	5	2	5		1											1 (126)		100
S3-RA1*...		6		5	12	11	1	56	5	1		2			2																		101
S3-RE1....	1 (58)			1	1	1		1				5	11	9	4	6	6	17	1	6	1	21	1	3		1		1		1	1 (105)		100

* Based on number of centromeres (*see* text).

strains sampled during this period, with the exception of S3-RA1. During the first 3 months of the investigation 43 cells, or over 66 percent of the first 65 cells analyzed in this strain, had 68 chromosomes. Since that time, and after the first instance of a dicentric chromosome was observed, an additional 36 cells have been scored. Among these, only 17 cells (approximately 47%) still retained the stemline number. Seven cells of this group were found to have a dicentric chromosome. The chromosome fragment resulting from the process of dicentric formation has apparently been lost.

The morphology of this dicentric appears to be the same in all 7 cells analyzed. This presumably means that the dicentric arose once as a result of a single reciprocal translocation and has been transmitted from this original cell to some of its progeny.

There was 1 cell in S3 with a possible dicentric of independent origin. However, this cell apparently did not transmit the dicentric to its progeny.

One clear spontaneous chromatid break giving a terminal deletion was also observed in 1 cell of S1 and is shown in figure 2.

Two cases of diplochromosomes were observed in S1 and S3-R3, probably resulting from the process of endoreduplication (*17*) during the previous interphase. This process apparently serves as one of the mechanisms of polyploidization (*18*).

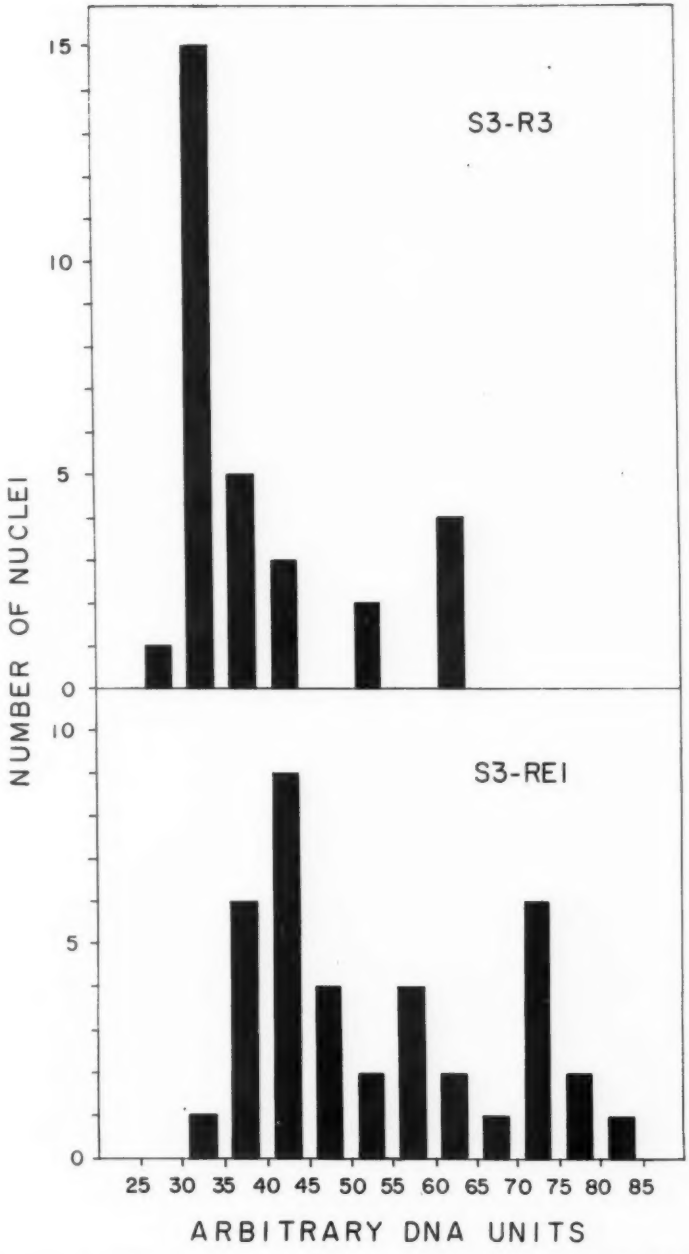
Cells with high chromosome numbers were also encountered in S3, S3-R3, and S3-RE1. This evidence, in addition to the multinucleated and giant cells (*see* B, p. 390), supports the view that polyploidization occurs in these clonal lines.

DNA Content

Since chromosome studies are possible only in cells capable of undergoing mitosis and in view of the degree of variability in chromosome number found in the clonal strains, it seemed desirable to measure quantitatively the DNA content in these clones so as to obtain a further independent estimate of the variability in these populations. Photometric measurements of individual Feulgen-stained nuclei were made on S3-R3, the most uniform clone, and S3-RE1, the most variable line as indicated by chromosome counts. Measurements were made of random samples of 30 and 39 nuclei in each line, respectively, with 2 replications each. The results of the measurements in terms of arbitrary DNA units are shown as histograms (text-fig. 2). In general, the distribution of nuclei appears to be remarkably similar to the results obtained by actual chromosome number determinations. Thus S3-R3, which has a stemline condition of low chromosome number, shows a similar distribution of DNA values, and this result is clearly distinct from the wider spread and higher DNA content found in S3-RE1. In both lines the peaks at the higher DNA values probably represent either higher polyploid cells or cells preparing for division.

Cell Division

A. *Mitotic activity.*—The frequencies of mitotic division for HeLa and its clonal derivatives were determined by recording the percentage of



TEXT-FIGURE 2.—Comparison of DNA measurements on individual nuclei in 2 HeLa clonal derivatives.

cells in mitosis each day, for 7 successive days after seeding, and they have been used as an estimate of growth rates. Cells with multipolar or anomalous spindles, anaphase bridges, and those undergoing possible endomitosis are included as cells in division. A random sample of at least 1,000 cells was counted from each culture per day. The results are presented in table 2.

TABLE 2.—*Mitotic indices (in percent) of HeLa and clonal cultures (based on over 1,000 cells per sample)—arrow indicates time of changing nutrient medium*

Days of culture	HeLa	S1	S3	S3-R3	S3-RA1	S3-RE1
1	2. 14	1. 79	1. 39	1. 29	1. 29	1. 10
2	3. 12	1. 77	3. 05	2. 27	2. 41	2. 12
3	4. 28	2. 26	3. 40	2. 63	3. 04	4. 90
4	4. 32	3. 20	3. 21	1. 65	3. 84	5. 10
⟶						
5	4. 02	4. 69	4. 82	3. 27	3. 69	4. 97
6	3. 72	4. 90	4. 80	3. 41	3. 25	4. 53
7	4. 01	3. 60	4. 06	2. 28	3. 84	3. 72

With approximately the same initial cell concentration per culture and under uniform culture conditions, these HeLa clonal derivatives appear to differ slightly from the parental HeLa strain and to a certain extent from each other as far as their mitotic activities are concerned.

It appears that growth rhythms vary among different strains under study. All strains with the possible exception of S3-RE1 appear to be slower in mitotic frequencies than HeLa. S3-R3 has the slowest rate. S1 seems to be a slow starter but eventually reaches the same rate as S3. Mitotic activities increase with the time of incubation and reach peaks in 3 or 4 days depending upon the strains.

B. *Abnormalities of dividing and nondividing cells.*—In general, visible cell abnormalities, both during mitosis and interphase were fairly common in HeLa and all of its clonal derivatives. It is, however, difficult to record quantitatively with absolute accuracy the relative frequencies of occurrence of various types of anomalies. Moreover, since all the observations were made on fixed and stained preparations, many dynamic features of abnormal division such as those observed by Hsu (*19*) with the use of cinematography were missed. Therefore, the frequency distributions of atypical dividing and nondividing cells in the strains, listed in table 3, represent the obvious irregularities and are thus only minimum values. For these comparisons daily records of each strain were pooled. Multipolar spindles are easily visible, yet the incidence found in these studies is lower than in Hsu's observations on HeLa. Comparatively few metaphases showed chromosomes arranged off the spindle, thus few anaphases showed lagging chromosomes. Also, few endomitotic cells were observed in these strains. In the absence of fragments, it is difficult to decide between sticky chromosomes and true bridges at anaphase. However, in strain S3-RA1, where a permanent dicentric

chromosome is retained, there seems to be little doubt about true bridge formation (fig. 4).

TABLE 3.—*Frequency distribution of abnormal dividing and nondividing cells in HeLa and its clonal cultures*

	HeLa	S1	S3	S3-R3	S3-RA1	S3-RE1
Mitotic abnormalities						
Tripolar spindle	5	6	7	3	6	18
Quadrapolar spindle	2	1	2	1	2	5
Pentapolar spindle	.....	1	3	.....	1	3
Hexapolar spindle	.....	.....	.....	.....	.....	1
Anomalous spindle	8	13	4	1	5	17
Endomitosis	2	2	2	.....	3	2
Bridges	1	1	.....	1	6	2
Total mitoses	282	236	264	166	238	287
Total atypical mitotic cells	18	24	18	6	23	48
Percent atypical mitotic cells	*6. 38*	*10. 17*	*6. 82*	*3. 61*	*9. 66*	*16. 72*
Others						
Nuclear constriction	4	2	3	4	7	12
Nuclear fragmentation	29	14	24	20	38	51
Nuclear budding	3	.....	1	1	.....	6
Multinucleated cell	10	7	7	5	11	14
Giant nucleus	9	20	6	2	1	5
Total cells examined	7661	7350	7420	6834	7683	7508
Total abnormal cells	73	67	59	38	80	136
Percent abnormal cells	*0. 95*	*0. 91*	*0. 79*	*0. 56*	*1. 04*	*1. 81*

In the case of nondividing cells, deviations from normal were also common. Many resting nuclei assume irregular shapes and sizes, with excessive lobing and fragmentation. A few instances of specific lobation with elongation of the nucleus and constrictions in its midportion, accompanied by a constriction of the cell membrane, were recorded. Instances of nuclear budding with knoblike protrusions from the nucleus were also observed (fig. 5). The final fate of these abnormal cells could not, of course, be determined in the fixed preparations. Multinucleated cells and giant cells (fig. 6) observed in these cell strains probably indicate the occurrence of polyploidy.

It is evident that abnormal dividing and nondividing cells occur with fairly high frequencies in both parental HeLa and its clonal derivatives. The frequency of mitotic abnormalities ranges from 3.61 percent in S3-R3 to 16.72 percent in S3-RE1. When all the atypical cells are considered, S3-R3 again represents the most stable strain, with 0.56 percent aberrations and S3-RE1 the most unstable, with 1.81 percent aberrations. Indeed, S3-RE1 seems to have a higher percentage of abnormal cells than does HeLa (0.95%).

Discussion

It is evident from the present studies that mutant clones derived from the HeLa strain are cytologically distinguishable from the parental strain and, in most instances, from one another. The wide chromosome-number variation reported by Hsu (*5*, *20*), together with the additional chromosome counts in this report, establish unequivocally that the parental

HeLa strain is a mixed population of variant cytological types. Gross morphological comparisons of cells further support this conclusion. Although our chromosome counts on HeLa are in general agreement with Hsu's, there is a shift of frequency distribution toward lower chromosome numbers. This difference can be explained on the basis of differences in the cultural history of the separate HeLa stocks, even though derived from the same original HeLa isolate. It is evident that modal chromosome numbers in such stocks may shift with time.

In contrast to the great heterogeneity in the parental HeLa strain, there is a notable uniformity of the chromosome numbers in 4 of the 5 clonal derivatives. All these clones can be distinguished from one another and from HeLa on the basis of chromosome number or by detailed comparative chromosome morphology, as in the case of S1 and S3 which have the same stemline number. The DNA content of different nuclei in the HeLa strain was shown (*20*) to be highly variable, a situation in close agreement with the actual chromosome counts. In the present studies, DNA measurements of S3-R3 and S3-RE1 also substantiate the results from direct chromosome enumeration.

The cytogenetical origin of the mutant characters detected in these clones of somatic mammalian cells is of considerable interest and significance. Puck and Fisher (*9*) have employed the term "mutant" in an operational sense to distinguish these clonal lines as isolates of divergent genetic constitution obtained from a single individual. They have also pointed out that such a designation contains no implications concerning the mechanisms by which such changes have arisen, their frequency, degree of reversibility, or randomness. Whereas the mutational changes in S1 and S3 are of spontaneous origin, it should be emphasized that the 3 clonal derivatives from S3 have been obtained from among radiation survivors.

It is not at present possible to exclude gene mutation as the mechanism responsible for the genetic variation found in the clonal lines studied. However, the foregoing observations indicate that changes in chromosomal constitution, especially in chromosome number, must be considered in addition to gene mutation as mechanisms potentially responsible for the mutant phenotypes of these clones.

The stemline condition generally present in such clones may explain the relative stability of their mutant traits. Puck and Fisher (*9*) reported that during the time of routine culture no change in any characteristic of either S1 and S3 had been observed. Further, single cell isolations (two from S1 and one from S3) were performed and both new isolates duplicate the behavior of the parental clonal stock from which they had originated. Although the present studies have shown that chromosome number variations can occur within these clones, new isolates may still be similar to their parental lines since they may represent either progenies from a cell having the original modal stemline number, or from a cell possessing the same mutant character even though carrying additional independent cytogenetical alterations.

The relatively high frequencies of abnormal dividing and nondividing cells in HeLa and in its clonal derivatives provide evidence as to mechanisms potentially responsible both for the origin of new mutant clones and for intraclonal chromosomal variations. It has been shown that both aneuploidy and polyploidy can arise within HeLa and its clones. Additional cytological evidence for endoreduplication, chromatid breakage, and dicentric formation, all of spontaneous nature, further indicate the instability of the clones. The data on abnormalities indicate a significant correlation between the frequencies of abnormalities and fluctuations in chromosome number within each clone. S3-R3 has been shown to be the most stable line in chromosome number and also to have the lowest aberration frequency. On the contrary, S3-RE1 appears to be the most unstable line on the basis of both these criteria. An inherently higher frequency of mitotic abnormalities in S3-RE1 may well account for the marked variability in chromosome number characteristic of this clone despite the fact that it is of relatively recent origin.

The mitotic index of HeLa estimated in these studies seems to differ from the observations by Hsu (*5*), especially in that he observed a very high frequency, close to 12 percent, in the early days of incubation. He further noted that the high mitotic indices dropped rapidly after a few days' incubation in the unchanged medium. It should be emphasized, however, that in his studies pieces of cell mass from old cultures were embedded in a plasma-embryonic extract clot to start a new culture. The fact that the cells were less disturbed during subculturing and that they were placed in immediate contact with rich nutrients may account for the surge of early mitotic activity. On the other hand, in our culture procedure cell dissociation by trypsinization, even though mild, may cause some trauma to cells, and some length of time would be required before the cells attach to the surface and resume normal growth. In addition, differences in media, incubation temperature, and other conditions may be responsible for these differences in mitotic rates. Nevertheless, under essentially the same conditions, it is reasonable to make interstrain mitotic frequency comparisons.

Studies on mitotic activities of these strains suggest that they differ in growth rates. With the present nutrient medium, S1 seems to be slower growing, at least at the beginning of incubation. This observation agrees with the results of Puck and Fisher (*9*) in which they have shown that S1 has a much lower plating efficiency than S3 in any concentration of human serum employed. Differences in mitotic activities among different clonal lines further imply genetic differences in their response to one specific nutritional and cultural environment.

Another interesting fact is that HeLa and S3-RE1 which are both mixed populations, show higher mitotic rates than the other 4 clones. Moorhead and Hsu (*21*) have shown in HeLa that the average duration for each phase of mitosis was longer for "aberrant" cells than for "nonaberrant" cells. These estimates of mitotic rates for HeLa and S3-RE1 may be higher than the actual case due to the possibly longer duration of

mitosis. However, the higher rates may be real, indicating a more effective cooperative-feeder system in a more heterogeneous population.

The conclusion to be drawn from the present observations, *i.e.*, that variant clonal lines can be obtained from a strain (such as HeLa) consisting of a heterogeneous population of cells in terms of chromosome numbers, is no doubt of general validity. Other similar studies (*22*) on clonal derivatives of mammalian cells (such as from Chang's human liver, conjunctiva, and appendix) have revealed a similar situation in that stemline conditions exist in most clones in contrast to the much wider variation in chromosome number found in the parental lines. Additional observations on S3 by Hsu (*20*) and on clones from mouse ascites tumors Krebs 2 and Ehrlich by Levan and Hauschka (*17*, *23*) support the same view.

Another more general implication of this and similar cytogenetical studies on mammalian cells in tissue culture should be indicated. It is, of course, of paramount importance in experimental biology to employ as nearly uniform material as possible so that repeated experiments will give comparable results. The HeLa strain has been employed in many types of research since its original isolation and establishment. The fact that this strain is cytologically heterogeneous and unstable may explain some of the different results obtained. Similar difficulties with other cell strains in continuous culture, such as the widely used mouse L strain, may stem from similar cytological abnormalities (*24*).

Although it is true that HeLa is of tumor origin and thus its derivatives may possess some features of malignancy and thereby exhibit cytological aberrations, recent evidence indicates clearly that changes in the characteristics of originally normal cell strains may also occur during long-term culture (*cf.* *25*, *26*).

For cytogenetical studies of mammalian cells *in vitro*, as, for example, the recovery of genetic variants and cytological studies of radiosensitivity, it is particularly desirable to employ normal diploid materials. However, recent evidence (*20*, *24*, *27*) has shown that most mammalian cell strains now in culture are heteroploids. Where such cell strains have to be employed, the use of pure clonal lines should theoretically give genetically uniform material. Indeed, the results of the present investigations have demonstrated that most isolated clones do exhibit more cytological uniformity than do the parental stocks. Nevertheless, present cytological evidence also shows that spontaneous chromosomal alterations, in both number and morphology, may arise in clones under long-term cultivation. These results indicate the desirability of practicing repeated plating and isolation within each clone, accompanied by constant cytological and genetical characterization, in order to maintain uniform cell lines for experimental purposes.

Summary

Comparative chromosomal studies have been made on HeLa, a strain of human cervical carcinoma, and 5 of its clonal mutant isolates.

In contrast to the great heterogeneity in the parental HeLa strain, there

is a notable uniformity of chromosome numbers in 4 of the 5 clonal derivatives. All these mutant clones can be distinguished from one another and from HeLa on the basis of chromosome number or by detailed comparative chromosome morphology.

Microspectrophotometric measurements of DNA content of individual nuclei in 2 clones show excellent agreement with actual chromosome enumerations.

These observations indicate that changed chromosomal constitutions, especially in chromosome number, must be considered in addition to gene mutation as mechanisms potentially responsible for the mutant phenotypes of these clones.

The occurrence of various mitotic abnormalities provides evidence as to the mechanisms responsible for variations in chromosome number found in HeLa and in its clonal derivatives. Studies on mitotic activities of the strains suggest that they differ in growth rates, which may imply genetic differences in their responses to specific nutritional and cultural environments.

Certain general implications of these studies are discussed as they apply to the use of mammalian cell cultures for various experimental purposes.

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PLATE 39

Idiogram analyses

FIGURE 1.—A cell from clone S3 with 78 chromosomes. The slight C-mitotic effect is due to colchicine pretreatment.

FIGURE 2.—A cell from clone S1 with 78 chromosomes. *Arrow* indicates a chromatid break.

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PLATE 40

FIGURE 3.—A cell from clone S3-RA1 showing 64 chromosomes and 1 dicentric chromosome. *Arrows* indicate positions of centromeres of the dicentric. Hypotonic-saline pretreated. Acetocarmine. × 2,700

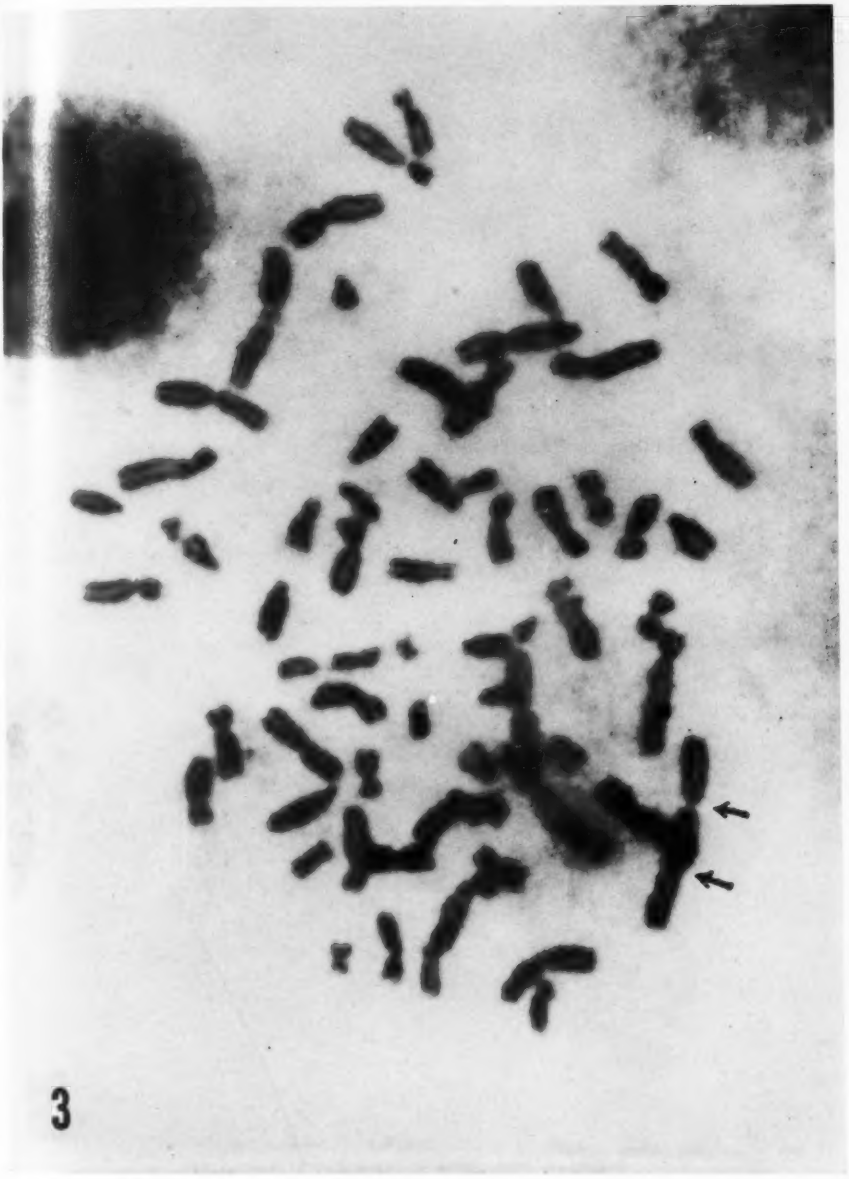
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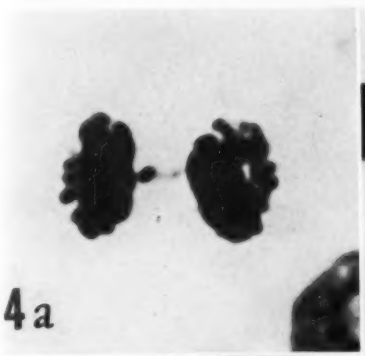
PLATE 41

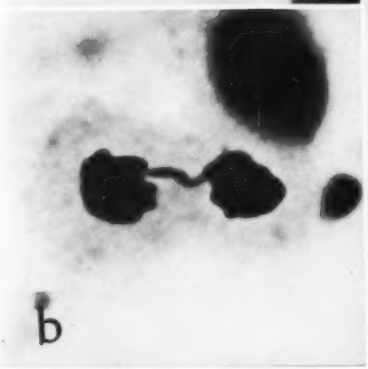
All figures from Feulgen-fast green stained preparations. X 1,520

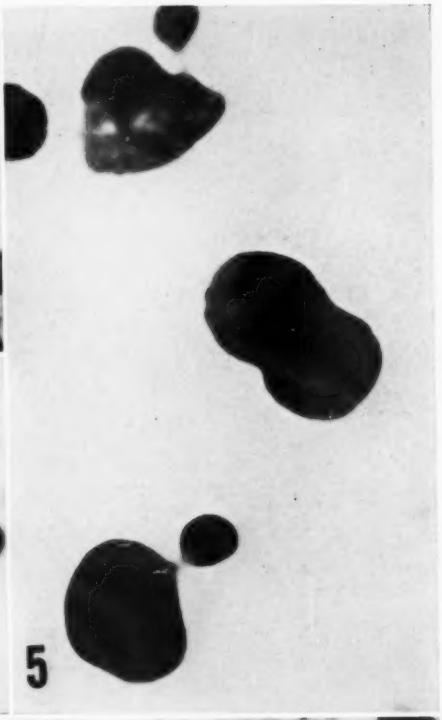
FIGURES 4*a* and *b*.—Two cells from clone S3-RA1 showing bridge formation during anaphase and telophase.

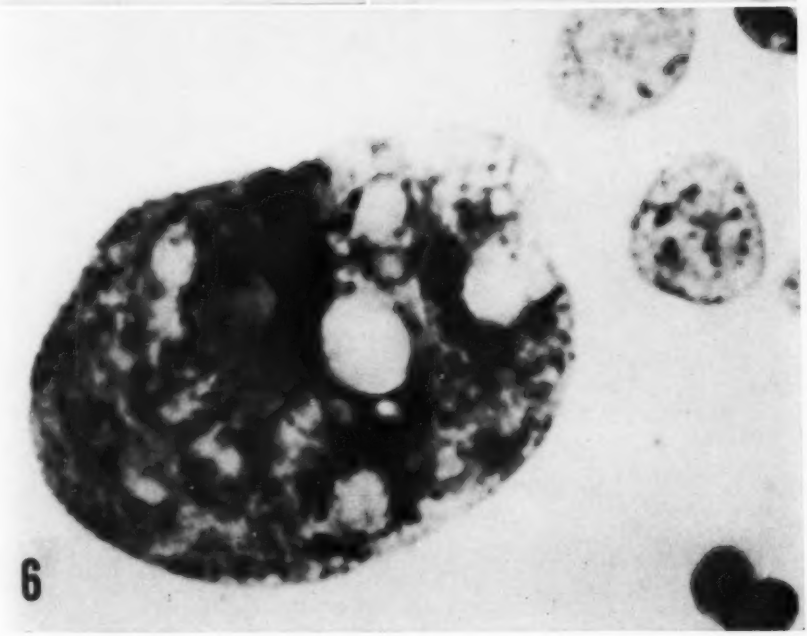
FIGURE 5.—Nuclei from a 3rd-day culture of clone S3-RE1 showing a nuclear budding with knoblike protrusions.

FIGURE 6.—A giant nucleus from a 3rd-day culture of clone S3-RE1 compared with nuclei of regular size.









450335—58——17

Glucose Utilization by Washed Mitochondria of Normal and Neoplastic Mouse Tissues [1, 2]

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It was reported from this laboratory in 1953 (*1*) that isolated, washed mouse brain mitochondria accumulated lactic acid and carried on a malonate-sensitive oxygen consumption with glucose as substrate. This demonstrates the presence on these particulates of both the glycolytic enzyme system and the Krebs cycle. In a subsequent paper (*2*), it was shown that mouse liver mitochondria did not use glucose *per se*, as determined above, but only its phosphorylated derivatives. This deficiency in enzymatic activity was paralleled by a deficiency in total phosphorus and total nitrogen content (*3*). In general, the results indicated that isolated brain mitochondria were more intact than liver mitochondria under the conditions used. The above data support the theory that all mitochondria *in vivo* carry the complete enzyme systems necessary for the energetic maintenance of the cell (*1*, *2*). Further evidence consistent with this interpretation is reported here, namely, that washed mitochondria, isolated from a number of other tissues by the same procedure used previously, were able to utilize glucose itself.

Materials and Methods

Normal brain, kidney, spleen, and testis tissues were obtained from adult white Swiss mice, N. I. H. stock. The Cloudman S91 melanoma (*4*) was carried in (BALB/c $\times$ DBA/2)F_1 mice by intramuscular transplantation. Tumor tissue, grown from about the twentieth generation of cytosieved implants, was used. The Krebs-2 carcinoma (*5*) was carried in N. I. H. white mice, an inbred strain of Swiss mice. The tumors from about the 40th transfer of the parent tumor were implanted intramuscularly in ascites form. Hepatoma 129 (*6*) was carried by subcutaneous transplant of solid pieces in C3H/HeN mice, and was in

[1] Received for publication August 7, 1957.

[2] The authors wish to express their thanks to Dr. Morris Belkin, Laboratory of Chemical Pharmacology, National Cancer Institute, for supplying the hepatoma 129, and to Mr. Richard V. Eck, Surgery Branch, National Cancer Institute, for the Cloudman S91 melanoma and Krebs-2 carcinoma used in these studies. They are greatly indebted to Mrs. Evelyn Peake, who performed the nitrogen analyses, and Mr. Charles Remsburg, who carried out the lactic acid determinations, both of the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

about the 60th generation when used. All tumors were harvested at maturity but before extensive necrosis had taken place. All animals were maintained on Purina laboratory chow.

Washed mitochondrial suspensions were prepared as previously described (*2*) from mouse brain, kidney, spleen, and testis and from transplants of the following mouse tumor strains: Cloudman S91 melanoma, Krebs-2 carcinoma, and induced hepatoma 129. Kidney, S91, and Krebs-2 tissues were homogenized in 0.375 *M* sucrose (*p*H 7.5) at a concentration of about 40 percent (by volume) of fresh tissue, testis and hepatoma at 30 percent, and brain and spleen at 20 percent. The centrifugal separability of the subcellular fractions of individual tissues was the main determinant of the homogenate concentrations used. The washed mitochondrial pellets from testis, hepatoma, and S91 were resuspended to contain the mitochondria from 1 ml. fresh tissue per ml. suspension, or 100 percent; similarly, the Krebs-2 suspension was 200 percent, kidney 130 percent, spleen 70 percent, and brain 40 percent. This variation was determined by the yield of mitochondria from the homogenates (small yield from Krebs-2, large from brain, etc.), since it is obvious that manometrically measurable activity is dependent on sufficient mitochondrial concentration. Oxygen consumption and nitrogen and lactic acid levels were measured as previously described (*2*, *3*).

Results

Table 1 exemplifies substrate utilization by brain and S91 melanoma fractions before and after washing the mitochondria and removing the "microsomes" from the supernatants. In both cases washing of the mitochondria tended to increase the Q values obtained with Krebs-cycle intermediates, and to lower those obtained with glycolytic substrates. None of the supernatants used oxygen significantly, either before or after clearance. It is interesting to note that the glycolytic activity of the mitochondria was decreased by washing off "soluble" material, as was that of the supernatant by removing the submicroscopic particle fraction by high-speed centrifugation. This suggests that both "microsome" fractions might simply represent material, not necessarily identical, from the mitochondria, derived by subsequent steps of the preparative procedure. Further, the findings are consistent with the view that the glycolytic enzymes are more easily separated from the mitochondrial structure than the Krebs-cycle enzymes.

Results of representative experiments with each tissue whose washed mitochondria used glucose are presented in table 2. The tissues are listed according to the rate at which their mitochondria utilized this substrate—brain being the most active and hepatoma the least. Unlisted experiments show that neither liver mitochondria (commonly used in subcellular research), nor those of kidney (active component of usual cyclophorase preparation), nor those of spleen used glucose in manometrically measurable amounts, when prepared and assayed as above. They did use fructose diphosphate (FDP). In all tissues studied, oxidation of both glycolytic

TABLE 1.—*Effect of washing mitochondria and clearing supernatants on metabolism of glycolytic and Krebs-cycle substrates**

Organ	Fraction	mg. N	Glucose		FDP		Pyruvate		α-Ketoglutarate		Endogenous	
			$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$
Brain	M	0. 81	6. 3	13. 6	4. 6	11. 2	5. 7	0. 6	3. 7	0	2. 7	0. 6
	WM	0. 60	4. 7	3. 5	4. 8	5. 3	9. 6	1. 3	9. 0	0	1. 6	0. 6
	S_1	0. 72	0. 4	32. 8	0. 3	19. 0	−0. 4	2. 9	−0. 2	0. 6	1. 3	0. 4
	S_2	0. 69	0. 2	22. 8	0	13. 4	0	−0. 1	−0. 1	0. 5	0. 9	0. 7
S91	M	0. 57	3. 7	2. 5	5. 0	6. 8	—	—	7. 5	—	0. 9	1. 0
		1. 02	—	—	—	—	2. 0	—	11. 4	—	1. 4	—
	WM	0. 51	1. 7	0	3. 2	2. 4	—	—	7. 2	—	0. 5	0. 8
		0. 72	—	—	—	—	3. 0	—	14. 7	—	0. 6	—
	S_1	0. 81	0. 6	31. 0	0. 6	22. 7	—	—	0	—	0. 6	0. 3
	S_2	0. 69	0. 3	30. 7	0	16. 2	—	—	0	—	0. 3	0. 5

*In each of the three experiments presented all fractions were derived from the same preparation. mg.N = nitrogen content of tissue fraction per vessel. M = unwashed mitochondria of a given homogenate. WM = equal aliquot of M, washed, and diluted like M. S_1 = primary supernatant (cleared of M). S_2 = equal aliquot of S_1, further cleared by recentrifugation. $Q_{O_2}^{(N)}$ = μM of oxygen consumed per hour per mg. N. $Q_{l.a.}^{(N)}$ = μM lactic acid accumulated per hour per mg. N. ΔQ values in columns 4–11 represent increase over the endogenous Q values given in the last 2 columns. The main compartment of each Warburg vessel contained, besides 0.3 ml. tissue fraction, 50 μM K_2HPO_4; 0.7 μM diphosphopyridine nucleotide; 40 μM niacinamide; 3.3–9.9 μM $MgSO_4.7H_2O$; 4 μM adenosine diphosphate; 4 μM adenosine triphosphate; 0.1–1.0 μM succinate ("sparker"); 0.02 μM cytochrome *c*. As substrate was added, 20 μM of either glucose, FDP (fructose diphosphate), pyruvate or α-ketoglutarate. Tissue and additions were made up in 0.375 *M* sucrose, *p*H 7.5, to give a final volume of 1.0 ml. per vessel. Center wells contained 0.1 ml. 2.0 *N* KOH. Temperature 38° C. Gas phase = air.

TABLE 2.—*Utilization of glycolytic and Krebs-cycle substrates by washed mitochondria and cleared supernatants of various normal and tumor tissues**

Organ	Fraction	mg.N	Glucose		FDP		Pyruvate		α-Ketoglutarate		Endogenous	
			$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$	$\Delta Q_{O_2}^{(N)}$	$\Delta Q_{l.a.}^{(N)}$
Brain	WM	0.60	4.7	3.5	4.8	5.3	9.6	1.3	9.0	0	1.6	0.6
	S_2	0.69	0.2	22.8	0	13.4	0	−0.1	−0.1	0.5	0.9	0.7
S91	WM	0.42	3.5	1.6	6.2	5.9	—	—	12.5	0.5	1.3	1.4
	S_2	0.87	−0.1	29.4	−0.1	16.7	—	—	0	0.3	0.4	0.7
Testis	WM	0.57	1.6	0.3	1.9	1.8	3.0	1.4	—	—	1.2	0.2
	S_2	0.57	0.3	1.9	0.5	10.5	0.4	0.4	—	—	0.2	0.8
Krebs-2	WM	0.51	0.8	0.9	3.6	3.0	4.3	—	8.0	—	0.6	0.5
	S_2	1.02	0.1	19.6	0.1	7.9	−0.2	—	—	—	0.3	0.6
Hepatoma	WM	0.45	0.5	0.4	3.7	3.9	0.9	1.0	—	—	0.5	0.3
	S_2	0.75	0.3	35.2	−0.3	12.2	−0.4	5.2	—	—	0.5	1.8

*See footnote, table 1.

and Krebs-cycle substrates took place only in the mitochondrial fraction.

The supernatants, as exemplified in table 2, accumulated lactic acid at characteristic rates, when glycolytic substrates were provided. These rates were independent of the rates found for the corresponding mitochondrial fraction (*1*). Liver, spleen, and kidney supernatants did not produce significant amounts of lactic acid from glucose, but did with FDP as substrate.

Discussion

It is apparent from the data presented that similarly prepared, washed, mitochondria from various tissues glycolyzed and respired at different rates with the same substrates. It is interesting to note that the mitochondria glucolyzed, without exception, only in the case of the neoplastic tissues reported. The relative rates of glucose utilization by the mitochondria of the tissues studied were, in general, paralleled by their relative rates of utilization of the other substrates. In this connection, the parallelism between glycolytic activity and chemical intactness of isolated mitochondria should be noted. As previously reported (*3*) mouse brain mitochondria survived the same isolation procedure in a more intact condition than liver mitochondria. This instability of liver mitochondria has a counterpart in that of hepatoma mitochondria, since the hepatoma mitochondria showed the lowest activity among those that glucolyzed, while the hepatoma supernatant glucolyzed freely.

It should also be emphasized that the substrate utilization of the supernatants of the tissues studied was paralleled qualitatively by their mitochondria. For instance, both hepatoma fractions used glucose and FDP, while the two liver fractions used FDP, but not glucose.

In general, the data indicate that the rate at which isolated mitochondria glycolyze and respire is dependent on their relative stability during isolation and washing, and that this stability is different for each tissue. The view, expressed previously (*1–3*), that *in vivo* both glycolytic and respiratory enzyme systems are bound to the mitochondria is supported by the enumeration of a graded series of washed mitochondria capable of using glucose.

Summary

Isolated mitochondria from mouse brain and the Cloudman S91 mouse melanoma were washed, and the corresponding supernatant fractions centrifugally cleared, in order to observe the effect of this treatment on the utilization of glucose, fructose diphosphate, pyruvate, and α-ketoglutarate. With these results as background, the use of the same glycolytic and Krebs-cycle substrates by washed mitochondria and cleared supernatants of normal tissues (brain, testis, liver, spleen, and kidney) and of neoplastic tissues (S91, Krebs-2 carcinoma, and hepatoma 129) was studied. The results allowed the enumeration of washed mitochondria in a series graded according to their ability to use glucose, with liver, spleen, and kidney unable to use glucose significantly. They are con-

sidered as additional evidence that *in vivo* all mitochondria carry the enzymes necessary for energetic maintenance of the cell.

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Note on the Increased Rate of Nail Growth in Mice Carrying the Milk Agent for Mammary Cancer,[1, 2]

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Structures that proliferate throughout the lifespan can be used to gauge the rate of formation of body protoplasm in general. The logic of utilizing a continually regenerating structure as an index of the "metabolic turnover" of the biochemist (*1*) is supported by the identical nature of growth and replacement when considered at a molecular level (*2*). Many scholars, including Muller (*3*), Gerard (*4*), and Needham (*2*) have emphasized the similarity of processes by which protein molecules are replicated, whether in the production of small units like enzymes or antibodies or in the reproduction of genes to form a cell or an entire organism.

Nails were selected for quantitative studies of regenerative rates that might serve as an index of the growth and replacement of tissues. Methodologic advantages of a technique based on nail growth include the following: (*a*) precision and accuracy of measurement; (*b*) availability for study at any age or continuously throughout the lifespan; (*c*) choice of intervals for study ranging from hours to months; (*d*) simplicity of technique, accessibility of nails for study, and lack of harm to subjects; and (*e*) a rough similarity in range of absolute values in several homeothermic species examined thus far (*5*).

Rates of nail growth have been found to reflect such varied physiologic states as aging in man and mouse (*6*, *7*), effects produced by long-continued partial starvation and by mitosis-inhibiting drugs in rats (*5*), certain forms of feeblemindedness such as Mongolism in man (*5*), and genetic differences between strains of mice (*7*). The present communication demonstrates, for 2 strains of mice, that rates of nail growth are also significantly altered in animals that carry the milk agent for mammary cancer.

Materials and Methods

The mice were young adult males of inbred strains BALB/c and C3H maintained in the laboratory of Dr. H. B. Andervont, of the National

[1] Received for publication August 19, 1957.

[2] Supported by the Harris McLaughlin Foundation and by a student fellowship provided by the Macy Foundation.

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Cancer Institute. Of 54 BALB/c animals, 27 carried the agent (*8*) and 27 were agent-free. Of 55 strain C3H animals, 27 carried the agent (*8*) and 28 were agent-free (*9*). Throughout this paper, a "+" sign denotes mice with the agent and a "—" sign denotes mice devoid of the agent.

In order to standardize the experimental conditions all mice were kept, 7 or 8 to a cage, in the same room, with experimental and control animals in adjacent cages. They were housed in plastic cages and given a bedding of wood shavings. An unlimited supply of Purina laboratory chow and tap water was available at all times, and all examinations were restricted to the month of July.

The following technique was used to ascertain the rate of nail growth. The nail of the middle digit of the right hind foot was used exclusively to avoid any differences between elongation rates of digits (*6*, *10*, *11*). A tattoo of carbon particles was used as a frame of reference. This was placed in the skin at the midline of the distal phalanx near the base of the nail plate. In order that much of the migration of the carbon particles would take place prior to the study of nail growth, the particles were introduced 24 to 72 hours beforehand. Migration of the particles continued at a slow pace for some time, however, and a sketch was made of the configuration of the tattoo before each period of study.

A fine cross-shaped incision was made with a razor blade exactly in the midline of the nail plate, and less than 1 mm. distal to its base. Movement of the incision was studied on only the proximal, relatively straight, portion of the nail plate. It was necessary to make a 2nd incision near the base of the nail plate before growth was measured for a 2nd week.

The distance between tattoo and incision was measured at a magnification of 40× with a Filar micrometer ocular. The micrometer was checked daily against a stage micrometer to ascertain whether there were changes in the optical equipment; the range of variation in number of Filar scale divisions per mm. of the stage micrometer was 3.845 to 3.857.

Readings were taken while the animals were anesthetized by an intraperitoneal injection of 10 percent sodium pentobarbitol (Nembutal ®) in 10 percent ethyl alcohol. The animal's leg was fastened to a board by adhesive tape applied immediately proximal to the web between the digits. The site to which the tape was applied and the manner of application were carefully standardized.

Each measurement of the distance between the incision on the nail plate and the stationary frame of reference was based on an average of 3 readings. The tape was removed, the animal readjusted, and the tape replaced before the 2nd reading was made. This procedure was again repeated before the 3rd reading was taken.

The intervals during which nail growth was measured extended for precisely 168 hours. After 7 days, readings of the distance between the tattoo and the incision on the nail plate were taken in the same manner as those immediately prior to this period of growth. The difference between the averages of readings for the 2nd and the 1st measurements is

the length of nail plate grown during the week. Values for nail growth represent elongation of the nail plate in mm. per 24 hours.

These studies were repeated for a 2nd period of 168 hours in order to check the accuracy of the original measurements. The mean value for rate of nail elongation in each animal is the average of 2 successive weekly periods of growth. Data were rejected for animals in which the values for 2 consecutive weeks did not agree within 0.02 mm.

Observations

In 3 animals (2 BALB/c and 1 C3H+) inordinate values were recorded for 1 week and not confirmed in the other week; the difference between the 2 weekly measurements was 0.033 to 0.039 mm. with a mean of 0.037 mm. In the remaining 106 mice the difference was less than 0.010 mm. in all but 4 animals. The average of the 2 weekly measurements in the 3 animals in question is believed to be in error, perhaps because of illness, damage to the nails, or faulty recording. Data for these 3 animals are not included in statistical analyses. If this data were included, the results reported below would be even more striking.

The data are summarized in table 1 and show that the absolute values for rates of nail growth in both strains were significantly higher ($P < 0.001$) in mice harboring the agent. Moreover, the relative values of nail growth per gram of body weight were also higher in infected animals.

Further analysis revealed that rates of nail elongation differed between the BALB/c + and BALB/c − groups to such an extent that there was no overlapping in their range of values, whereas with the C3H groups there was a small overlap. Further work is necessary to establish the significance of this difference between strains.

The clear-cut results of this experiment permit presentation of an earlier experiment performed during July, 1955, and in which cruder methods for measuring nail growth were used. Indeed, the significant results obtained in this earlier study encouraged further exploration of the problem.

Two groups of strain C3H mice (9 with and 9 without the agent) were used and the findings are summarized in tables 2 and 3. Table 2 shows the rates of nail growth in individual mice. Table 3 shows the results in groups and is presented for comparison with data included in table 1. It is seen that the range of values for nail growth in individual mice was distinct with the exception of one mouse (table 2), and the values recorded for groups (table 3) are comparable to those of the later study. However, the rate of elongation of nails in relation to body weight was not significantly greater in infected mice. The higher mean weight of infected mice could have been responsible for this difference. For the convenience of the reader, table 4 is presented in which findings with strain C3H mice of both experiments are compared.

The coefficient of variation (C.V. in percent) in values for rate of nail elongation in 2 successive weeks was 1.6 for C+, 2.6 for C−, 1.5 for C3H+, and 3.0 for C3H− mice. The differences in C.V. between agent-

TABLE 1.—*Rate of nail growth in inbred mice carrying or free of the milk agent for mammary cancer*

Strain	Milk agent	No. of mice	Age (*days*)*				Body weight (*gm.*)*			
			Mean	S. D.	Range	Median	Mean	S. D.	Range	Median
BALB/c	+	26	117. 2	7. 88	106–127	119. 0	30. 3	1. 88	27–33	30. 0
BALB/c	−	26	109. 2	2. 61	106–113	109. 5	30. 3	1. 96	28–34	30. 0
C3H	+	26	116. 7	2. 77	112–122	118. 0	30. 2	1. 91	27–33	30. 0
C3H	−	28	120. 9	0. 96	118–122	121. 0	31. 4	2. 36	28–35	31. 5

Strain	Milk agent	No. of mice	Rate of nail growth (*mm./24 hrs.*)					Rate of nail growth (*mm./24 hrs. per gm. of body weight*)				
			Mean	S. D.	Range	Median	*P*†	Mean	S. D.	Range	Median	*P*†
BALB/c	+	26	0. 1034	0. 00206	. 0980–. 1070	. 1035	<. 001	. 00342	. 000233	. 0031–. 0038	. 0035	<. 001
BALB/c	−	26	0. 0928	0. 00180	. 0865–. 0955	. 0935		. 00307	. 000161	. 0028–. 0034	. 0031	
C3H	+	26	0. 1034	0. 00210	. 0965–. 1065	. 1035	<. 001	. 00343	. 000177	. 0032–. 0039	. 0034	<. 001
C3H	−	28	0. 0922	0. 004029	. 0840–. 1030	. 09275		. 00295	. 000229	. 0026–. 0035	. 0029	

*At end of the first of the 2 successive weekly periods when nail growth was measured.
†*P* values apply to differences between the means.

TABLE 2.—*Data for nail growth in individual mice, obtained in early study with less refined methodology*

Strain	Milk agent	Age (*days*)	Body weight (*gm.*)	Rate of nail growth (*mm./24 hrs.*)	
				Absolute values	Relative values (*mm./24 hrs. per gm. of body weight*)
C3H	+	118	36	.085	.0024
		120	34	.102	.0030
		118	33	.103	.0031
		120	32	.104	.0032
		120	33	.104	.0032
		120	33	.108	.0033
		118	33	.109	.0033
		118	33	.109	.0033
		118	34	.114	.0034
C3H	−	115	32	.088	.0028
		117	34	.088	.0026
		110	27	.089	.0033
		110	29	.090	.0031
		117	27	.091	.0034
		115	31	.091	.0029
		110	28	.091	.0032
		116	30	.092	.0031
		110	28	.093	.0033

positive and agent-negative animals was significant in both C ($P = .007$) and C3H ($P = .001$) strains.

Discussion

This discussion is concerned only with the first experiment presented herein because better techniques were employed.

The investigation was carried out under identical environmental conditions and with inbred animals similar in sex, size, age, and body weight, in order to compare the effect of the agent on the rate of nail growth. Furthermore, the agent was in a species that is its natural host, a situation in which host-agent relationships tend to be well adapted.

Values for nail growth were almost identical for the 2 inbred strains: both in absolute terms and relative to body weight; in the extent of increase in agent-carrying animals; and in the degree of variation in the same animal in successive weeks.

The experiment provided the following strong evidence that the agent promoted increased rates of elongation of nails: 1) Statistically significant differences between groups of agent-containing and agent-free mice that were of the same genetic derivation, in good health, and kept under identical environmental conditions; 2) almost distinct ranges of values in infected and noninfected groups; 3) similar data for 2 inbred strains in which the nature, magnitude, and uniformity of the effects were almost identical. It is concluded that infection with the mammary-tumor agent of mice is associated with sustained increase in elongation rates of nails.

TABLE 3.—*Effects similar to those in table 1, but observed in an earlier study with another group of male C3H mice, utilizing less refined techniques for study of nail growth*

Strain	Milk agent	No. of mice	Age (*days*)*				Body weight (*gm.*)*			
			Mean	S. D.	Range	Median	Mean	S. D.	Range	Median
C3H	+	9	118. 9	0. 61	118–120	118	33. 4	1. 73	32–36	33
C3H	−	9	113. 3	3. 84	110–117	115	29. 6	2. 06	27–34	29

Strain	Rate of nail growth (*mm./24 hrs.*)					Rate of nail growth (*mm./24 hrs. per gm. of body weight*)				
	Mean	S. D.	Range	Median	*P*	Mean	S. D.	Range	Median	*P*
C3H+	0. 1042	0. 00831	. 085–. 114	. 104	<. 001	. 00313	. 000101	. 0024–. 0034	. 0032	<. 72
C3H−	0. 0903	0. 00253	. 088–. 093	. 091		. 00308	. 000247	. 0026–. 0034	. 0031	

TABLE 4.—*General comparability of data obtained with early, cruder technique and that with later, more refined procedures*

Method used in study of nail growth	C3H− mice						C3H+ mice					
	No. of mice	Mean age (*days*)	Mean weight (*gm.*)	Nail growth (*mm./24 hrs.*)			No. of mice	Mean age (*days*)	Mean weight (*gm.*)	Nail growth (*mm./24 hrs.*)		
				Mean	S. D.	Range				Mean	S. D.	Range
Early (crude)	9	113	30	. 090	. 003	. 088−. 093	9	119	33	. 104	. 008	. 085−. 114
Later (more refined)	28	121	31	. 092	. 004	. 084−. 103	26	117	30	. 103	. 002	. 096−. 106

Summary

It is concluded that infection with the mammary-tumor agent of mice is associated with sustained increase in elongation rates of nails.

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Influence of Antigenic Factors in the Production of Antitumor Cytotoxic Sera[1]

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The application of immunologic techniques to the study of cancer has been reviewed in the excellent articles of Woglom in 1929 (*1*), Spencer in 1942 (*2*), and Hauschka in 1952 (*3*). The earlier studies were concerned largely with the therapeutic and diagnostic potentialities of the immunologic method. Later studies placed more emphasis on the importance of the genetic, chemical, and morphologic properties of antigens in normal and neoplastic tissues. This gradual evolution of immunologic studies resulted from the recognition of the complexity of tissue as an antigen and was fostered by the development of inbred strains of animals and improved methods of chemical and morphologic fractionation of the cell.

As a result of these methods for antigenic simplification, immunologic techniques provide a valuable means not only for evaluating some of the basic differences between various normal tissues but also for determining antigenic differences between normal and abnormal tissues.

Previous reports indicate that heterologous antitumor sera exert a cytotoxic effect on tumor cells *in vitro* (*4–6*). In the present experiments various antigenic factors (species and strain of animal, type of organ, and neoplastic and normal tissue) were studied to determine their relative importance in the production of the antitumor cytotoxic effect. The antigenic properties of morphologic and chemical cell fractions were also investigated.

Materials and Methods

Preparation of Antigens

Homogenates.—Tissues were homogenized with equal volumes of 0.85 percent saline solution in a Potter-Elvehjem homogenizer for 2 minutes. Strain C3H/He mice were used as the source of spontaneous mammary carcinoma, lactating mammary gland, and spleen tissue. Transplanted mammary tumor MT7 (*without* the milk agent), transplanted mammary tumor C3HBA (*with* the milk agent), and transplanted hepatoma 98/15 were obtained from strain C3Hf/He mice. Hepatoma 10 and normal liver tissue were obtained from strain BALB/c mice. Homogenates were

[1] Received for publication September 5, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

also prepared from transplanted rat mammary tumor #1643 and from family #13 guinea-pig kidney.

Tissue fractions.—Mitochondrial, microsomal, and *soluble protein* fractions were isolated from spontaneous C3H/He mammary carcinoma by the sucrose technique (*7*).

The *nucleoprotein* fraction was isolated from spontaneous C3H/He mammary carcinoma by the method of Mirksy and Pollister (*8*).

A preparation of the *anticatalase fractions* from transplanted mammary tumor C3HBA was obtained from Greenfield and Meister (*9*).

Preparation of Antisera

Prior to injection into the test animals, each of the freshly prepared antigens was mixed with an equal volume of mineral oil and 1/10 volume Arlacel A[3] by repeated aspiration and ejection with a syringe. Two or more rabbits were injected subcutaneously with each antigen once a week for 3 weeks; each rabbit received a total of 15 mg. nitrogen per kg. body weight. Antisera were harvested 1 week after the last antigen injection. Serum collected from each rabbit before immunization was used as the control for antiserum from the same rabbit. All sera were stored at −20° C., between tests. Equal volumes of sera from all rabbits receiving the same antigen were pooled for both the preimmunization control tests and the tests with immune sera.

One of the test antisera was prepared in strain C57BL mice by 4 weekly injections of 0.1 ml. of a 12.5 percent C3HBA mammary-tumor saline homogenate. The antiserum was harvested 1 week after the last injection and tested against mammary tumor C3HBA by the method described for the cytotoxic test.

Cytotoxic Test

Transplanted mammary carcinoma C3HBA was prepared in the following manner and used to test the cytotoxic activity of the antisera in all the experiments. The tumor tissue was passed through a tissue press into 0.85 percent saline solution and was further broken up by repeated aspirations through hypodermic needles of decreasing caliber from #18 through #25. Following this aspiration, the cell suspension was centrifuged for 10 minutes at 600 × g. After withdrawal of the supernate and the layer of red blood cells, the tumor cells were resuspended in 0.85 percent saline solution and recentrifuged. The supernate was again removed and the cells were measured and resuspended in a known volume of 0.85 percent saline solution to give the desired concentration of tumor cells. Examination of the final cell suspension in a red blood cell counting-chamber revealed that the cells were separated except for very occasional clumps of 3 to 4 cells. The counts revealed that the number of cells in all suspensions prepared in this way were constant, yielding approximately 5,000 cells per 0.1 ml. of a 0.4 percent cell suspension.

In the actual *in vitro* test system, the final saline supernate was with-

[3] Kindly provided by the Atlas Powder Company, Wilmington, Del.

drawn and replaced with the quantity of antiserum necessary to produce the desired cell concentration. The antiserum-tumor cell suspension was incubated for 2 hours at 37° C., and was thoroughly mixed at half-hour intervals during the incubation period.

Preliminary experiments indicated that 0.1 ml. of 0.4 percent suspension of C3HBA tumor cells in normal rabbit serum inoculated subcutaneously in normal male and female strain C3Hf/He mice, produced tumors in 80 to 100 percent of the hosts. All of the tumors developed within less than 60 days after transplantation. Hence, in the experimental studies, the strain C3Hf/He test mice were observed for tumor growth for not less than 80 days after the subcutaneous implantation of 0.4, 0.8, 1.6, and 3.2 percent cell suspensions incubated with the various test antisera. Several separate determinations were performed on each of the test antisera and the results obtained for like serum were combined to minimize the effect of chance variation in the tumors or mice.

In one test series, immune serum was heated at 56° C. for 30 minutes, combined with an equal volume of saline, and incubated at 37° C. for 2 hours with the various concentrations of C3HBA mammary-tumor cell suspensions. In another series, immune serum heated at 56° C. for 30 minutes was combined with an equal volume of preimmune serum before incubation with the tumor cells. Both groups were implanted and observed as described in the previous paragraph.

If no tumors developed in the mice receiving transplants, the animals were retransplanted subcutaneously with 0.1 ml. of a freshly prepared 0.8 percent C3HBA mammary-tumor suspension and observed for another 80 days to determine whether or not the host mice possessed immunity to the tumor transplant.

Observations

All of the test sera were evaluated in terms of their ability to inhibit the growth of transplanted mammary tumor C3HBA in homologous strain C3Hf/He mice. In all cases, cytotoxic activity was determined on the basis of the number of tumors arising in the strain C3Hf/He mice after the implantation of various concentrations of C3HBA mammary-tumor brei previously incubated with immune or preimmune serum from the same rabbit. *Cytotoxic potency was expressed as the concentration of tumor cells necessary to overcome the inhibiting effects of the antiserum.* The results were analyzed both in terms of the cytotoxic potency of each antiserum as compared with its preimmune counterpart and in terms of a comparison between the various antisera.

Because of the inherent variation in the cytotoxic activity of the preimmune sera, the concentration of C3HBA mammary tumor implanted was standardized so that the control animals would develop less than 100 percent tumors (the actual average control tumor incidence was 91.6%), thus reflecting more minute differences in the effectiveness of the antiserum.

Table 1 shows the relationship between the various immune sera and their preimmune counterparts. With the exception of the cytotoxically

TABLE 1.—*Tumors developed* in strain C3Hf/He mice transplanted subcutaneously with C3HBA mammary tumor incubated with normal rabbit sera and antisera prepared against various tissue homogenates*

Test tissue—antigen	Serum†	Percentage of tumor cells in serum-tumor mixture	No. of tumors developed‡	Percentage of tumors developed	D ± SE§	*P*	Antigens common to the test tissue and C3HBA mammary tumor‖			
							Species	Organ	Strain	Tumor
C3Hf/He MT7 mammary tumor homogenate	Preimmune	0. 4	15/16	94			X	X	X	X
	Immune	0. 4	2/16	13	81 ± 10. 4	0. 010				
	"	0. 8	5/16	31	63 ± 13	0. 010				
	"	1. 6	7/16	45	49 ± 14	0. 010				
	"	3. 2	11/16	69	25 ± 13	0. 05				
BALB/c mammary tumor homogenate	Preimmune	0. 4	27/31	87			X	X	—	X
	Immune	0. 4	3/16	19	68 ± 12	0. 01				
	"	0. 8	5/16	31	56 ± 13. 4	0. 010				
	"	1. 6	12/15	80	7 ± 12. 8					
	"	3. 2	16/16	100						
C3H lactating mammary-gland homogenate	Preimmune	0. 4	57/68	84			X	X	X	—
	Immune	0. 4	18/40	45	39 ± 9	0. 010				
	"	0. 8	28/40	70	14 ± 8. 5	0. 1				
	"	1. 6	38/39	97						
	"	3. 2	40/40	100						
C3H hepatoma 98/15	Preimmune	0. 4	13/18	72			X	—	X	X
	Immune	0. 4	5/18	28	44 ± 15	0. 01				
	"	0. 8	6/13	46	26 ± 17. 5	0. 1				
	"	1. 6	5/18	83						
	"	3. 2	18/18	100						
C3H spleen homogenate	Preimmune	0. 4	23/23	100			X	—	X	—
	Immune	0. 4	6/16	38	62 ± 12. 1	0. 010				
	"	0. 8	15/15	100						
	"	1. 6	15/15	100						
	"	3. 2	15/15	100						

BALB/c liver homogenate	Preimmune	0. 4	16/16	100			X	–	–	–
	Immune	0. 4	6/8	75	25 ± 15. 6	0. 2				
	"	0. 8	8/8	100						
	"	1. 6	8/8	100						
	"	3. 2	8/8	100						
BALB/c hepatoma 10 homogenate	Preimmune	0. 4	15/16	94			X	–	–	X
	Immune	0. 4	12/16	75	19 ± 12. 4	0. 2				
	"	0. 8	16/16	100						
	"	1. 6	16/16	100						
	"	3. 2	15/16	94						
Rat mammary-tumor homogenate	Preimmune	0. 4	15/16	94			–	X	–	X
	Immune	0. 4	15/16	94						
	"	0. 8	16/16	100						
	"	1. 6	16/16	100						
	"	3. 2	16/16	100						
Guinea-pig kidney homogenate	Preimmune	0. 4	13/16	81			–	–	–	–
	Immune	0. 4	15/16	94						
	"	0. 8	16/16	100						
	"	1. 6	16/16	100						
	"	3. 2	16/16	100						
C3HBA mammary-tumor homogenate, IN C57BL mice	Preimmune	0. 4	14/16	88			X	X	X	X
	Immune	0. 4	16/16	100						
	"	0. 8	16/16	100						

*The term "tumors developed" is used to mean the development of a progressively palpable tumor within 80 days from the date of transplantation.

†Preimmune serum represents serum from rabbits before immunization was begun; immune serum represents serum from the same rabbits after immunization.

‡The numerator is the number of animals that developed tumors and the denominator is the total number of animals transplanted with the serum-tumor mixture.

§Difference between preimmune and immune percentages ± the standard error of the difference.

‖ Symbol X indicates the test tissue and the C3HBA mammary tumor both possess this antigen. Symbol – indicates the test tissue does not posess this antigen in common with C3HBA mammary tumor.

inactive, spontaneous, C3H mammary-tumor homogenate antiserum prepared in strain C57BL mice, the test sera were all prepared in rabbits. As shown by this table, which lists the tissue antigens in the order of decreasing potency of their antisera, C3Hf/He mammary tumor MT7 (*without* the milk agent) brei proved to be the most powerful in inhibiting the growth of mammary tumor C3HBA. Inhibition was also obtained with antisera prepared against BALB/c mammary-tumor homogenate, C3Hhepatoma 98/15, C3H la ctating mammary gland, C3H spleen homogenate, BALB/c liver homogenate, and BALB/c hepatoma 10. No inhibition was obtained with antisera prepared against rat mammary-tumor homogenate or with guinea-pig kidney homogenate. Therefore, it is apparent that the cytotoxic potency of the various antisera decreased in direct relation to the decrease in the number of antigens common to both the test tissue and the C3HBA mammary-tumor brei. It is also clear that only heterologous antisera were cytotoxically active in this *in vitro* cytotoxic test system.

In the second experimental series the cytotoxic potency of antisera against several fractions of a spontaneous C3H mammary tumor were evaluated in relation to the inhibition obtained with preimmune serum from the same rabbits. Greater tumor inhibition was observed with antisera against the microsomal fraction than was noted with antisera against the tumor homogenate. Less cytotoxically potent than the tumor homogenate, but still capable of inhibiting the growth of mammary tumor C3HBA in strain C3Hf/He mice, were the mitochondrial, nucleoprotein, and soluble protein fractions. Antiserum against the anticatalase fraction showed no cytotoxic activity. In this series the materials used as antigens were derived from a single source, spontaneous C3H mammary tumor, and shared a common species, strain, organ, and tumor background and differed only in morphologic nature. The relatively greater cytotoxic potency of the antisera against the microsomal fraction therefore suggest a concentration of the antigenic factors important in cytotoxicity in this fraction. The statistical evaluation of the cytotoxic activity of these tissue fractions is given in table 2, which lists the antigens in the order of their decreasing cytotoxic potency.

In another comparison the cytotoxic potency of each of the antisera described was evaluated in relation to the effectiveness of other antisera, rather than in relation to the effectiveness of its own preimmune serum. For this analysis,[4] the antiserum against spontaneous C3H/He mammary-tumor homogenate was selected as the "control" against which the other antisera were compared since the C3H/He mammary-tumor homogenate antiserum most closely resembles the species, strain, organ, and malignant characteristics of the C3HBA mammary tumor. In the following outline the antisera are arranged in decreasing order of their cytotoxic potency as compared with the cytotoxic effectiveness of the "control" antisera:

[4] We are indebted to Mr. M. A. Schneiderman, of the National Cancer Institute, for the statistical analysis.

TABLE 2.—*Tumors developed* in strain C3Hf/He mice transplanted with C3HBA mammary tumor incubated with normal rabbit sera and antisera prepared against various tissue fractions*

Test tissue fraction—antigen	Serum†	Percentage of tumor cells in serum-tumor mixture	No. of tumors developed ‡	Percentage of tumors developed	D ± SE§	*P*
Spontaneous C3H mammary-tumor microsome	Preimmune	0. 4	24/24	100		
	Immune	0. 4	4/16	25	75 ± 10. 8	0. 010
	"	0. 8	6/16	39	61 ± 12. 2	0. 010
	"	1. 6	11/16	69	31 ± 11. 5	0. 01
	"	3. 2	15/15	100		
Spontaneous C3H mammary-tumor homogenate	Preimmune	0. 4	39/45	87		
	Immune	0. 4	10/36	28	59 ± 9	0. 010
	"	0. 8	12/24	50	37 ± 11. 4	0. 010
	"	1. 6	18/24	75	12 ± 10. 2	0. 1
	"	3. 2	15/16	94		
Spontaneous C3H mammary-tumor mitochondria	Preimmune	0. 4	27/28	96		
	Immune	0. 4	4/24	17	79 ± 8. 6	0. 01
	"	0. 8	10/16	63	33 ± 12. 7	0. 01
	"	1. 6	13/16	81	16 ± 11. 4	0. 2
	"	3. 2	15/16	94		
Spontaneous C3H mammary-tumor nucleoprotein	Preimmune	0. 4	20/25	80		
	Immune	0. 4	. 6/20	30	50 ± 13	0. 010
	"	0. 8	8/15	53	27 ± 15. 2	0. 1
	"	1. 6	14/16	88		
	"	3. 2	16/16	100		
Spontaneous C3H mammary-tumor soluble protein	Preimmune	0. 4	16/16	100		
	Immune	0. 4	12/16	75	25 ± 10. 8	0. 02
	"	0. 8	16/16	100		
	"	1. 6	16/16	100		
	"	3. 2	16/16	100		
C3HBA mammary tumor anticatalase (impure)	Preimmune	0. 4	7/7	100		
	Immune	0. 4	6/6	100		
	"	0. 8	7/7	100		
	"	1. 6	7/7	100		
	"	3. 2	7/7	100		

*, †, ‡, § *See* footnotes table 1.

1) Definitely higher activity (statistically significant from control at *P* 0.05):
   C3H mammary tumor (MT7) homogenate antiserum
2) Intermediate group (not statistically different from control):
   a) Higher activity—spontaneous C3H mammary-tumor microsomal fraction antiserum
   b) Equal activity—spontaneous C3H mammary-tumor mitochondrial fraction antiserum. BALB/c mammary-tumor homogenate antiserum
   c) Lower activity—spontaneous C3H mammary-tumor nucleoprotein fraction antiserum

3) Definitely lower activity (statistically significant at P 0.05 or less):
C3H lactating mammary-gland antiserum
BALB/c hepatoma 10 homogenate antiserum
C3H spleen antiserum
BALB/c liver homogenate antiserum
C3H mammary-tumor soluble protein fraction antiserum
BALB/c hepatoma homogenate antiserum

The role of complement in the *in vitro* cytotoxic test was investigated by heating C3H mammary-tumor homogenate antisera at 56° C. for 30 minutes before incubation with the C3HBA tumor homogenate. As shown in table 3, the strong cytotoxic effect of this antiserum was markedly reduced by heating, presumably due to destruction of the complement. The cytotoxic activity of the antiserum was partially restored by the addition of preimmune rabbit serum before incubation with the C3HBA mammary-tumor homogenate further confirming the role of complement in the cytotoxic test.

TABLE 3.—*Cytotoxic activity of C3H spontaneous mammary-tumor antisera heated at 56° C. for 30 minutes*

Percentage of tumor cells in serum-tumor mixture	Immune serum and saline		Preimmune serum and saline		Immune serum, heated at 56° C. for 30 minutes, and saline		Immune serum, heated at 56° C. for 30 minutes, and preimmune sera	
	No. of tumors developed*	Percentage of tumors developed	No. of tumors developed*	Percentage of tumors developed	No. of tumors developed*	Percentage of tumors developed	No. of tumors developed*	Percentage of tumors developed
0.4	0/21	0	6/19	32	4/19	21	1/14	7
0.8	1/21	5	11/13	85	13/19	68	5/16	31
1.6	5/20	25	21/21	100	19/20	95	8/13	61
3.2	17/21	81	19/19	100	18/20	90	13/14	93

*The term "tumors developed" is used to mean the development of a progressively palpable tumor within 80 days from the date of transplantation.

Two hundred and eighty-three of the mice inoculated with C3HBA mammary-tumor homogenate combined with various antisera failed to develop tumors. Subsequently, these mice were reinoculated with 0.1 ml. of an 0.8 percent C3HBA mammary-tumor cell suspension to test for host immunity. In all cases the retransplanted group developed tumors that appeared at approximately the same time and developed at the same rate, which indicated that host immunity was not responsible for the failure of the original tumor implant and also that no host immunities resulted from the original tumor implant.

Discussion

In view of numerous reports indicating that the injection of tissue into a heterologous host results in an antiserum that inhibits the growth of

cells ("cytotoxic" properties), the present study was designed with the twofold purpose of 1) evaluating the relative importance of species, strain, organ, and tumor-specific antigens in the production of a cytotoxic antiserum and 2) determining whether these cytotoxin-producing antigens were associated with particular cellular components.

Since the isolation and independent study of a single antigen is extremely difficult in the inherently complex protein substance of a tissue, an attempt was made to control as many of the variables as possible in the test system. For this reason, all of the antisera were tested for their capacity to inhibit the growth of a single tissue so the evaluation of the cytotoxic potency of the test antigens could be made against the constant antigenic properties of the target tissue.[5] More specifically stated, in the present study the cytotoxic effectiveness of each of the antisera was evaluated in terms of its capacity to inhibit the growth of a neoplastic *mouse* tissue, originating in the *mammary gland* of *strain C3H/He* mice.

Thus, by carefully controlling the genetic factors and by statistically evaluating the variation in cytotoxic activity resulting from the variation of a single group of factors in the test antigen, *i. e.*, species, organ, etc., it is possible to determine the relative importance of the species, strain, organ, and neoplastic-tissue antigens in the present cytotoxic test system. By a similar comparison it is also possible to compare the cytotoxic potency of various cellular components.

In the first test series contrasting the immune with the preimmune serum obtained from the same rabbit, it is apparent that cytotoxic potency decreases in direct relation to decrease of the number of common antigens shared by the test antigen and the target tissue. The most marked inhibition was obtained with test antigens that most closely resembled the target tissue in all major respects—species, strain, organ, and neoplasia. Of these major antigenic groupings, species antigens appear to predominate as evidenced by the absence of cytotoxicity in rat mammary-tumor antiserum and guinea-pig kidney antiserum. The nature of this antigenic difference has never been clearly demonstrated but its existence as one of the distinguishing characteristics of different species of animals is well known. The importance of these species-specific antigens is further suggested by the observation that C3H mammary-tumor homogenate, an excellent antigen for the production of cytotoxic antiserum in rabbits, failed to produce a cytotoxic antiserum when injected into C57BL mice. Possibly the ineffectiveness of the antiserum prepared in mice reflected a weak ability of the mouse to produce antibodies, though the probability that the cytotoxic effect was absent because the host, the test antigens, and the target tissue possessed numerous common antigens seems the more likely explanation.

Considering again the mouse-rat relationship it might also be postulated that the process of conversion from normal to neoplastic tissue involves certain antigen changes and that the apparent contrast between rat and

[5] For simplicity "test antigen" refers to the material against which the antisera were prepared; "target tissue" to the C3HBA mammary tumor.

mouse mammary-tumor homogenate antisera was less dependent upon species differences than it was upon the alteration or deficiency of unknown antigenic factors in either the rat or mouse tumor tissue. That this postulate would not, however, serve as an adequate explanation for the cytotoxic ineffectiveness of guinea-pig kidney antisera, again stresses the major role of the species antigens.

Species antigens alone, however, did not result in maximum cytotoxic potency and their effectiveness was increased by the concomitant presence of one or more of the other major antigenic groups. Organ-specific antigens were a significant factor in the cytotoxic phenomenon as evidenced by the relatively higher cytotoxic potency of antisera against normal and neoplastic mammary tissue from mice of either the same or a different homologous strain as compared with the potency of antisera obtained from other mouse organs. These organ antigen differences, clearly demonstrable in the same species, were obliterated in studies on different species. Rat mammary tumor, differing from the target tissue in species of origin only, was completely ineffective as a cytotoxin, suggesting perhaps that organ-specific antigens may be inextricably bound and, to a certain extent, determined by the more predominant species-specific antigens.

Also under the influence of the predominant species-specific antigens, but significant in its own right as well, is the greater cytotoxic potency of tumor tissue as compared with normal tissue. In all cases, antisera against mammary-tumor homogenate, whether obtained from C3H/He, BALB/c, or MT7 mammary tumors, inhibited the growth of the target tissue mammary tumor C3HBA more markedly than did antiserum against strain C3H/He normal lactating mammary gland. To term this response as a "tumor-specific" antigen in the usual sense would, however, be a misnomer since both organ specificity, as demonstrated by the hepatoma-mammary tumor comparison, and species specificity as demonstrated by the rat mammary tumor-mouse mammary tumor contrast, appeared to supersede in importance the antigenic factors associated with normal as compared with neoplastic tissues. All other antigenic factors being equal, however, it is apparent that the neoplastic as compared to the normal form of the test antigen enhances the cytotoxic effectiveness of the resulting antisera aginst a neoplastic target tissue.

Strain antigens as demonstrated by Gorer (*10*) in his histocompatibility studies were not detectable in the *in vitro* cytotoxic test system, since strain differences between the test antigens and the target tissues resulted in no apparent difference in cytotoxic potency of the antisera. Perhaps the use of other inbred strains or other target tissues would have accentuated the importance of strain antigens which were, in the BALB/c mammary-tumor homogenate–C3H/He mammary-tumor homogenate contrast, of no apparent significance. Strain difference was also of secondary importance in determining the cytotoxic potency of antisera against BALB/c liver and hepatoma.

An attempt was made to demonstrate whether antigenic simplification by cell fractionation would reveal something of the intracellular nature of the antigens responsible for the cytotoxic property. When these anti-

sera against cellular fractions (microsomal, mitochondrial, nucleoprotein, soluble protein, and anticatalase) were evaluated in terms of their cytotoxic potency as compared to the potency of their preimmune counterparts, a relative concentration of the cytotoxic activity in the microsomal fraction similar to that previously reported in mouse leukemia by Dulaney (*11*) was observed. The anticatalase fraction was inactive and the other cell fractions less active than the C3H mammary-tumor homogenate from which they were derived. Whether this relative concentration of cytotoxically active antigenic factors in the microsomal fraction would also be demonstrable if a comparison of microsomal fractions from different species, organ, strain, neoplastic and normal tissues were made, presents an interesting problem.

The possibility that the Forssman antigen was involved in the production of these cytotoxic antisera was ruled out by the observation that guinea-pig kidney antiserum was cytotoxically inactive against the C3HBA mammary-tumor target tissue.

Antisera heated at 56° C. for 30 minutes also showed a marked decrease in cytotoxic potency which was in part restored by the addition of unheated preimmune rabbit sera, suggesting that complement was of importance in the cytotoxic effect.

That the mammary-tumor milk agent did not play a significant role in the cytotoxic effect of the antisera to C3H tissues is evident when it is noted that the C3H mammary tumor MT7 without the agent produced one of the most potent cytotoxic antisera.

Absorption studies were not done on any of the antisera since it appeared that nonspecific reactions resulting from absorption with as complex an antigen as tissue would have obscured rather than clarified the relationship of the various antigenic factors in the *in vitro* cytotoxic test system, particularly since these antigenic factors appeared to be tangentially dependent upon each other.

The observation that a favorable combination of a number of antigenic factors results in a greater total response than might be anticipated from the sum of each of the individual factors indicates a cumulative cytotoxic effectiveness and suggests the possibility that these factors each enhance the antigenic potency of the other. Perhaps this apparent mutual interdependence is primarily a reflection of the effect of administering an antigen not yet pure in the chemical sense. On the other hand, it may be more dependent upon the possibility that fundamentally certain tissue proteins exist not as entities but rather in relation to each other and that they must therefore be examined as a group and cannot be evaluated as isolated substances. In either case, however, it is apparent that a valid comparison of cytotoxic potency can be made only in those instances in which strict control of the antigenic variables is exercised.

Summary

Immune sera prepared in rabbits against rat mammary tumor, guinea-pig kidney tissue, and a variety of normal and neoplastic mouse tissues,

were tested *in vitro* for their ability to inhibit the growth of transplanted mammary tumor C3HBA in strain C3Hf/He mice. The degree of tumor inhibition, or cytotoxic potency, obtained with each of these immune sera was evaluated 1) in relation to the effectiveness of its preimmune counterpart and 2) in relation to the effectiveness of the other antisera.

Both of these analyses revealed that species-specific antigens were predominant in producing the cytotoxic sera. Organ-specific antigens were also of major importance in cytotoxicity in a homologous species but were not demonstrable between heterologous species. Comparisons of antisera prepared against tumor tissues and against normal tissues, when both were derived from the same species and organ as the target tissue (C3HBA mammary tumor), revealed that tumor antigens enhance the cytotoxic potency. This enhancement of cytotoxicity was not demonstrable with tumors differing from the target tissue in organ of origin in a homologous species. Strain-specific antigens were not demonstrable in the *in vitro* cytotoxic test system.

Fractionation of spontaneous strain C3H/He mammary-tumor tissue revealed that the microsomal fraction was more active cytotoxically than either the tumor homogenate or the other tissue fractions. The mitochondrial, nucleoprotein, and nuclear and soluble protein fractions were less active cytotoxically than the tumor homogenate. The anticatalase fraction of tumor tissue was cytotoxically inactive.

Forssman antigen was not involved in the production of a cytotoxic sera; the presence of complement was, on the other hand, essential to cytotoxic activity. Retransplantation of C3HBA mammary tumor into test animals that failed to develop tumors indicated that the original tumor implant induced no host immunity.

The cytotoxic potency of each antisera was roughly proportional to the number of antigenic factors common to both the test and the target tissue. A favorable combination of antigenic factors in the test tissue resulted in a total cytotoxic effect greater than that anticipated on the basis of the sum of the individual antigenic factors.

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Induction of Hepatomas in Strain C3H Mice with 4-*o*-Tolylazo-*o*-Toluidine and Carbon Tetrachloride [1]

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Investigations of the factors involved in the occurrence and development of hepatomas in experimental animals have contributed significantly to knowledge of the cancer process. The scope of these investigations and the progress achieved during recent years are best appreciated by reading reviews written during 1947 (*1*) and 1955 (*2*). There are good reasons for continued interest in experimental hepatomas. The normal liver provides sufficient control material for chemical studies and it has been studied intensively by anatomists, cytologists, and enzymologists. Moreover, its responses to exogenous stimuli have received considerable attention (*2*).

Hepatomas occur spontaneously in laboratory animals (*3*) and can be induced by a variety of agents (*2*). The importance of chemical carcinogens (*2*, *4*), hormonal stimulation (*5*, *6*), and diet (*7*, *8*) in their occurrence and growth has been established by numerous investigators. The literature concerning these tumors is large and has been reviewed by authors of the preceding references. Further references will be restricted to investigations in which either inbred mice, especially those of strain C3H, or 2 hepatoma-inducing compounds, an azo dye (4-*o*-tolylazo-*o*-toluidine) and carbon tetrachloride, were used, because this paper reports the production of hepatomas in inbred strain C3H mice by administration of these compounds.

Spontaneous hepatomas occur in inbred strains of mice (*9–11*) but strains C3H and CBA have received the most consideration (*9*). In these strains the tumors are found in older animals and occur more frequently in males. Experimental evidence indicates that the incidence can be altered by hormonal stimulation (*9*, *10*, *12*) and by diet (*13*).

Induced hepatomas have attracted the attention of investigators for many years. Valuable reviews of the work with carcinogenic azo dyes have been prepared by Shear (*14*), who described the studies leading to their discovery, by Kinosita (*15*, *16*), and by Kirby (*17*, *18*), who sum-

[1] Received for publication September 26, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

marized the results of experiments with mice up to 1945. Hepatomas have been produced in various inbred strains of mice by the administration of azo dyes (*18*). In this laboratory, mice of strains A, BALB/c, I, C57BL, and C3H have received 4-*o*-tolylazo-*o*-toluidine as crystals moistened with glycerol (*19–23*), as a solution in olive oil (*24*, *25*), and as a dietary constituent (*26*). Hepatomas occurred in all strains following exposure to large amounts of the carcinogen but in one study (*25*) a single subcutaneous or oral dose of 4 or 8 mg. dissolved in olive oil induced hepatomas in strain A females. In every experiment where both sexes of the same strain were used, females were more susceptible than males to the induced tumors and in one (*23*), designed to explore sex difference in BALB/c mice, intact females were much more susceptible than intact males, castrate males were more susceptible than intact males, and testosterone propionate increased the resistance of castrate males and females.

Strain C3H mice were not used in early studies of sex susceptibility because the females developed mammary tumors at an early age and a line free of the mammary-tumor agent was not available. In 3 experiments (*19–21*) a total of 56 C3H males were used and in another (*24*), which included only 8 females and 9 males, all the females developed mammary tumors with only 2 surviving to the end of the experiment. Thus, a sex variation in susceptibility of strain C3H animals to azo-dye-induced hepatomas could not be determined in these experiments.

A high incidence of mammary tumors at an early age in strain C3H females was probably the chief reason why they were not included in the early work on hepatoma production by oral administration of carbon tetrachloride. Edwards (*27*, *28*) discovered that this compound produced hepatomas in mice and found that strain C3H males, in contrast to their resistance to 4-*o*-tolylazo-*o*-toluidine, were susceptible to carbon tetrachloride. It is also of interest that in these investigations, and others (*29*), when both sexes of the inbred strains A, Y, and C57L were used, the authors did not record a sex difference in response, while in one (*28*), BALB/c females exhibited a high incidence of induced hepatomas. Moreover, Eschenbrenner's (*30*, *31*) interesting series of dose-response studies with carbon tetrachloride in strain A mice did not reveal a sex difference in susceptibility.

This brief review of findings with 4-*o*-tolylazo-*o*-toluidine and carbon tetrachloride permits the conclusion that female mice are more susceptible than males to hepatomas induced with 4-*o*-tolylazo-*o*-toluidine, whereas the sexes are probably equally susceptible to hepatomas induced with carbon tetrachloride. Because these conclusions were derived from results obtained with inbred mice that develop few spontaneous hepatomas it was considered of interest to ascertain whether similar sex differences in response to the carcinogens could be detected with strain C3H mice, which have a high incidence of spontaneous hepatomas. It was found that, in common with other inbred strains, C3H females were more susceptible to 4-*o*-tolylazo-*o*-toluidine and both sexes were susceptible to carbon tetrachloride.

Materials and Methods

Strain C3H mice have been maintained in this laboratory since 1930 and, unless free of the mammary-tumor agent, females show a high incidence of mammary tumors (*32*). Both agent-carrying and agent-free mice (*33*) were used in this investigation to ascertain whether the presence of the agent influenced their susceptibility to induced hepatomas. Only nonbreeding animals were used. They were kept in the same room and fed an unlimited diet of Purina laboratory chow and tap water.

Carbon tetrachloride was dissolved in olive oil and administered orally by means of a stomach tube. A fresh solution was prepared for each injection and food was removed from the animals the evening before receiving the solution. Crystalline 4-*o*-tolylazo-*o*-toluidine (hereafter called azo dye) was moistened with glycerol and administered subcutaneously in the right axillary region by means of a 12-gauge trocar (*23*). The concentration of carbon tetrachloride and amount of azo dye used are given in the description of individual experiments.

Histologic sections were prepared from all hepatomas and from many livers showing gross signs of reaction to the carcinogens. These latter lesions are designated as liver changes and consisted of an enlarged roughened liver containing few or numerous cystic areas. The macroscopic and microscopic appearance of liver changes and hepatomas have been described previously (*21*, *28*).

Experimental Procedures and Results

Two experiments were performed, the results of which are summarized in table 1.

In the first experiment, carbon tetrachloride was administered orally to agent-free and agent-carrying strain C3H mice of both sexes. The females were 3.5 to 6.5 months old and the males 3.5 to 7.5 months old when the experiment was started. The first 2 doses of carbon tetrachloride consisted of 0.2 cc. of a 2 percent olive-oil solution at an interval of 1 week. The following week the concentration was raised to 3 percent which was given, at weekly intervals, for 17 weeks. Thus, animals surviving the treatment each received 19 weekly injections; the first 2 of a 2 percent solution, and 17 of a 3 percent solution.

The results are shown in the table. Males with or without the agent gave similar results, which are combined. Findings in females are not combined because many of those harboring the agent developed breast tumors. These females were of interest because 13 that developed mammary tumors were killed when 8 to 10 months old, and before they had received the entire series of injections. One had developed a hepatoma and 9 showed definite hepatic changes; the 3 without lesions were autopsied when 8, 9, and 10 months old. Detection of gross lesions in these mice was the reason for killing 28 others when they were 11 months of age and only 1 to 2 months after receiving the last dose of carbon

TABLE 1.—*Occurrence of hepatomas in strain C3H mice following administration of carbon tetrachloride or 4-o-tolylazo-o-toluidine**

Sex†	No. of mice	Carcinogen	Mice without hepatomas / Mice with hepatomas — Age in months								Totals	Tumor incidence (*percent*)	Average number of hepatomas in tumor-bearing mice
			8	9	10	11	12	13	14	15			
Males	77	Carbon tetrachloride	—	1/0	0/2	5/10	5/24	2/6	3/16	0/3	16/61	79	1. 8
Females+	42	Carbon tetrachloride	3/0	3/0	6/1	6/2	9/7	2/1	1/1	—	30/12	29	1. 2
Females−	37	Carbon tetrachloride	—	—	—	3/2	9/4	3/5	5/6	—	20/17	46	1. 5
Males	53	4-*o*-tolylazo-*o*-toluidine	—	—	—	—	2/0	—	1/1	20/29	23/30	57	1. 8
Females−	34	4-*o*-tolylazo-*o*-toluidine	—	—	—	—	4/0	—	0/1	6/23	10/24	70	2. 9
Males	45	None	—	—	—	—	2/0	—	—	21/22	23/22	49	1. 3
Females−	30	None	—	—	—	—	2/0	—	2/0	25/1	29/1	3	1. 0

*Strain C3H males show hepatoma incidences between 12 and 55 percent when 15 months old; breeding females show incidences between 5 and 11 percent when over 15 months of age (*9*).
†A + sign denotes agent-carrying and a − sign agent-free females.

tetrachloride. Of these 28 mice, 14 had hepatomas and 6 of the remainder showed pronounced liver changes.

The surviving animals were killed as shown in the table, and in each group the incidence of hepatoma was higher than that found in untreated strain C3H mice (*9*). The occurrence of breast tumors in females with the agent accounts for their lower incidence of hepatomas and it is reasonable to assume that the incidences in the other groups would have been higher if all the animals had been kept until 15 months of age.

All mice with hepatomas had definite liver changes and, of the 16 males, 30 agent-containing females, and 20 agent-free females that did not develop hepatomas, 8, 13, and 9, respectively, showed hepatic changes. This relationship between hepatic changes and hepatomas should be kept in mind when hepatomas induced with the azo dye are discussed.

The outcome of this experiment showed clearly that administration of carbon tetrachloride to strain C3H mice induced hepatomas in both sexes.

In the second experiment, 2 to 6 months old agent-free and agent-containing males and agent-free females were given subcutaneous injections of the azo dye. Untreated litter mates served as controls for the occurrence of hepatomas. The azo dye was prepared for administration by moistening 1 gm. of the crystalline compound with 30 drops of glycerol. Each mouse received approximately 10 mg. at 4-week intervals, for a total of 7 injections, or 70 mg. of the compound.

The results are shown in the table. Six experimental and 4 control mice were killed when they were 12 months of age. None had developed a hepatoma and only 1 female showed slight liver changes. The remaining animals were killed and autopsied as seen in the table. A hepatoma incidence of 70 percent in the experimental females showed conclusively that the azo dye evoked the hepatomas. All females, including the 6 that were free of hepatomas at 15 months of age, had developed pronounced hepatic changes. All of the control females had normal livers and the one tumor-bearing animal had a single hepatoma, whereas the experimental females with hepatomas averaged 2.9 tumors per mouse. The number of hepatomas ranged between 1 and 7 in these animals.

Agent-free and agent-carrying males gave similar results and are combined in the table. Those exposed to the azo dye showed a hepatoma incidence of 57 percent and the controls an incidence of 49 percent. A difference of 8 percent is not considered significant because it is known that different groups of strain C3H males show considerable variation in their incidences of spontaneous hepatomas (*9*). The average number of hepatomas in tumor-bearing mice was higher in the experimental group; the range in both groups was from 1 to 3 hepatomas.

The absence of liver changes in males was of special interest. The livers of all control and experimental males were grossly normal with the exception of one in the experimental group.

The results of this experiment show that, in confirmation of earlier findings with inbred mice, strain C3H females are far more susceptible than males to hepatomas induced with the azo dye.

Discussion

The pronounced sex differences in susceptibility of inbred mice to both spontaneous and induced hepatomas should be of some assistance to those interested in the sequence of events leading to malignancy, because in such animals host variations in susceptibility are reduced to the minimum. In strains showing a high incidence of spontaneous hepatomas, males are more susceptible than females and there is some evidence that castration or estrogenic stimulation alters the susceptibility of males.

When the azo dye, 4-*o*-tolylazo-*o*-toluidine, is administered to inbred strains, the females are more susceptible than males to induced hepatomas and this sex response can be altered by castration or exposure to sex hormones. Furthermore, it is known that a single subcutaneous or oral dose of 4 or 8 mg. of the azo dye dissolved in 0.25 cc. of olive oil induces hepatomas in strain A females. This should also be of some interest to those interested in the mechanism of hepatoma induction, because large amounts of a carcinogen may elicit cytologic and metabolic reactions that are not involved in the occurrence of tumors.

When carbon tetrachloride is administered to inbred strains, both sexes respond by developing induced hepatomas.

The findings presented here show that all the above-mentioned variations can be studied within a single inbred strain of mice. Males of strain C3H have been used to demonstrate the effects of castration, hormonal stimulation, and diet upon the occurrence of spontaneous hepatomas (*9-13*). In view of the greater susceptibility of females to hepatomas induced with the azo dye it is reasonable to assume that, similar to strain BALB/c (*23*), the sex response of strain C3H can also be altered by castration or administration of sex hormones. The susceptibility of strain C3H animals of both sexes to hepatomas induced with carbon tetrachloride completes the sex pattern revealed by other inbred strains. Thus within one inbred strain, males are more susceptible to the occurrence of spontaneous hepatomas, and females are more susceptible to hepatomas induced with an azo dye, while both sexes are susceptible to hepatomas induced with carbon tetrachloride.

Determination of the minimum amount of azo dye and carbon tetrachloride required for the production of hepatomas in strain C3H females is the only additional information needed for their use in studies on the mechanisms of hepatoma induction.

Conclusion

An azo dye, 4-*o*-tolylazo-*o*-toluidine, and carbon tetrachloride were administered to strain C3H mice that were devoid of the mammary-tumor agent.

Females were more susceptible than males to hepatomas induced with the azo dye, while both sexes were susceptible to hepatomas induced with carbon tetrachloride.

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Announcements

Nomenclature of Cell Strains

At the International Tissue Culture Meeting, held in Glasgow on August 26 and 27, 1957, the subject of the nomenclature of cell strains used in tissue culture was considered. After general discussion, a committee was appointed and recommendations were made. It was suggested that authors should give the following information when first mentioning a cell strain in the course of a publication:

1) Whether the tissue of origin was normal or neoplastic and, if neoplastic, whether benign or malignant.
2) Whether the tissue was adult or embryonic.
3) Animal species of origin.
4) Organ of origin.
5) The cell type (if known).
6) The designation of the strain.
7) Whether the strain has been cloned and, if so, the clone number.
8) The reference to the original article in which the strain was described.

It was further suggested that the designation of the strain should consist of a series of not more than four letters indicating the laboratory of origin, followed by a series of numbers indicating the strain, *e.g.*, NCL 123. Thus, a strain might be described: Normal adult rat heart fibroblast, strain NBG 111, clone 29 (Lobachevsky, Z.: J. Metaphys. Cytol. 99: 77, 1922). Subsequently, in the same communication it would suffice to refer to the strain by its strain designation.

Survey of compounds, Supplement I

Supplement I to *Survey of Compounds Which Have Been Tested for Carcinogenic Activity* is now available. The publication lists 981 compounds which were tested during the period 1948 through 1953. Data were collected and classified by Professor Philippe Shubik, of the Chicago Medical School, and by Dr. Jonathan Hartwell, of the National Cancer Institute. Of the total number tested, 779 are reported for the first time in these tests. Copies of the new publication are available from the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. Price per copy $3.50.

ERRATA

Journal of the National Cancer Institute

Volume 19, Number 4, October 1957

In the discussion that follows the paper entitled "Histogenesis in Plant Tissue Cultures," by Gautheret:

Page 590. Remarks by Dr. Riker; *for* "July, 1956" *read* "July, 1954."

Volume 19, Number 5, November 1957

In the paper entitled "Cocarcinogenic Activity of Cholesterol Oxidation Products and Sesame Oil," by Bischoff:

Page 986, Plate 84, Figure 6, Second Line of Legend: *for* "sesamin concentrate" *read* "sesamolin concentrate."

O

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i. e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

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Published by the U. S. Department of Health, Education, and Welfare in accordance with section 220, title 44, U. S. Code

Use of funds for printing this publication approved by the Director of the Bureau of the Budget, May 14, 1956

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1958

For sale by the Superintendent of Documents, U. S. Government Printing Office Washington 25, D. C. - Price per copy $2.00

Subscription price for two volumes per year in the United States, Canada, and Mexico, $20.00; foreign, $25.00

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